Plant Activity and Organic Contaminant Processing by Aquatic Plants

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Plant Activity and Organic Contaminant Processing by Aquatic Plants

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This work is deadicated to my family

I have always relied on their friendship, love, and support
as the stable platform from which I can reach for the stars

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2,3,4,6-TeCP	2,3,4,6-tetrachlorophenol
2,3,5-TCP	2,3,5-trichlorophenol
2,3,5-TFP	2,3,5-trifluorophenol
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,4,5-TCP	2,4,5-trichlorophenol
2,4,6-TBP	2,4,6-tribromophenol
2,4,6-TCP	2,4,6-trichlorophenol
2,4-D	2,4-diphenoxyacetic acid
2,4-DCP	2,4-dichlorophenol
2,6-DCP	2,6-dichlorophenol
2-Cl-3,5-DFP	2-chloro-3,5-difluorophenol
2-Cl-4-FP	2-chloro-4-fluorophenol
2-CP	2-chlorophenol
3,5-DFP	3,5-difluorophenol
4-Cl-2-FP	4-chloro-2-fluorophenol
4-CP	4-chlorophenol
99Te	technetium-99
α	plant activity
β	relative plant activity

BCF	bioconcentration factor
C_0	initial concentration
$C_{0,\ fitted}$	initial concentration fit to contaminant uptake data
C_{aq}	mass of contaminant in aqueous phase
C_i	mass of contaminant internal to plants
cm	centimeters
C_p	mass of contaminant enzymatically processed by plants
CPR_{50}	critical plant residue
C_s	mass of contaminant sorbed to plants
d	days
DCPAG	2,4-dichlorophenyl-b-D-glucopyranisyl-(6-1)-b-D-apiofuranoside
DCPG	2,4-dichlorophenyl-b-D-glucopyraniside
DCPMG	2,4-dichlorophenyl-b-D-(6-O-malonyl)-glucopyraniside
DDA	2,2-bis(p-chlorophenyl)acetic acid
DDMU	1-chloro-2,2-bis(p-chlorophenyl)ethylene
DDT	dichlorodiphenyltrichloroethane
EC_{50}	50% effect concentration
f	fraction of contaminant in protonated form
$f_{cytosol}$	fraction of contaminant in the protonated form in the cytosol
$F_{unprocessed}$	fraction of contaminant internal to plants not enzymatically processed
FW	fresh weight
GC	gas chromatography
HCA	hexachloroethane

HPLC	high performance liquid chromatography
hr	hours
Hz	hertz
IUPAC	International Union of Pure and Applied Chemistry
k	contaminant uptake rate coefficient
K_f	Freundlich parameter
K_H	Henry's Law constant
K_m	Michaelis-Menton half velocity constant
K_{OC}	organic carbon partioning coefficient
K_{OM}	organic matter partioning coefficient
K_{ow}	octanol water partition coefficient
LC_{50}	concentration of toxin that is lethal to 50% of organisms
LD_{50}	toxic dose that is lethal to 50% of organisms
LFER	linear free energy relationship
LOEC	lowest observed effect concentration
MCI	molecular connectivity index
min	minutes
mm	millimeters
MS	mass spectroscopy
MW	molecular weight
n	Freundlich parameter
NMR	nuclear magnetic resonance
NOEC	no observed effect concentration

P	plant mass
PCBF	plant bioconcentration factor
PCE	tetrachloroethene
PCP	pentachlorophenol
ppm	parts per million
q_e	mass of contaminant sorbed
QSAR	quantitative structural activity relationship
RCF	root concentration factor
rpm	revolutions per minute
RSD	relative standard deviation
S	aqueous phase concentration
SOM	soil organic material
sp.	species
T	tesla
t	time
$t_{1/2}$	half-life
T_b	boiling temperature
TCD	thermal conductivity detector
TCPAG	2,4,5-trichlorophenyl-b-D-glucopyranisyl-(6-1)-b-D-apiofuranoside
TCPG	2,4,5-trichlorophenyl-b-D-glucopyraniside
TCPMG	2,4,5-trichlorophenyl-b-D-(6-O-malonyl)-glucopyraniside
TFA	trifluoroacetic acid
T_m	melting temperature

TNT	2,4,6-trinitrotoluene
TSCF	transpiration stream concentration factor
USEPA	United States Environmental Protection Agency
UV/Vis	ultra-violet/visable wavelengths
UV_{212}	ultra-violet wavelength 212
WET	whole effluent testing

SUMMARY

The fate of organic contaminants in aquatic plant systems was explored through (i) experimental development of relationships to describe sorption, uptake and enzymatic processing of contaminants and inhibition of aquatic plants by contaminants and (ii) incorporation of experimental relationships into a conceptual model to address contaminant fate in aquatic plant systems. This study focused on interactions of aquatic plants *Lemna minor* and *Myriophyllum aquaticum* with halogenated phenols, with special emphasis placed on 2,4,5-trichlorophenol (2,4,5-TCP) and 2,4-dichlorophenol (2,4-DCP). These chlorinated phenols are precursors for the highly toxic and heavily applied herbicides 2,4,5-T and 2,4-D and were examined in detail. Direct land application of halogenated phenols or their precursors has resulted in contamination of seepline areas, wetlands and surface waters. In addition, halogenated phenols are highly toxic and are generally resistant to microbial degradation, properties which limit microbial remediation options as effective alternatives for the reclamation of contaminated sites.

Relationships for fundamental interactions between plants and contaminants that dictate uptake, enzymatic processing and sequestration of contaminants by aquatic plants were investigated. An assay which quantified production of oxygen by plants was developed to quantify plant metabolic activity and examine inhibition by contaminants. Uptake of 2,4,5-TCP was linearly correlated with metabolic plant activity, where uptake rate increased with an increase in metabolic plant. In addition, contaminant uptake rate linearly increased with

fraction of contaminant in protonated form in aqueous phase, demonstrating that contaminant speciation was a critical factor in determining contaminant uptake rate. Therefore, plant activity, contaminant pK_a and media pH were established as critical parameters controlling rate of contaminant uptake. Fifteen chlorinated and fluorinated phenols were examined to assess effects of chemical and physical properties on rate of uptake and enzymatic processing. When plant activity and concentration of contaminant in protonated form were not variables, contaminant pK_a had a significant effect on rate of contaminant uptake. Parameters such as octanol-water partition coefficient and molecular connectivity index were not well correlated with contaminant uptake rate indicating that contaminant hydrophobicity was not a controlling factor in uptake rate by aquatic plants.

A conceptual model was developed which incorporated plant activity and inhibition into a mathematical description of uptake of organic contaminants by aquatic plants. Abiotic partitioning into plants, equilibrium sorption to plants and enzymatic processing by plants were described by a series of differential equations. The conceptual model was parameterized using experimental data delineating effects of contaminant speciation, plant activity, and inhibition by contaminants on contaminant uptake. The model was verified using independently gathered data, and contaminant uptake rates for uninhibited and inhibited systems were well described by the model.

Experimentation with radio-labeled chlorinated phenols established that contaminants were sequestered internal to plants by plant enzymatic processing and no metabolites were released into aqueous phase. ^{19}F NMR was established as a technique to quantify transformation and conjugation products internal to plants and contaminant assimilation by plants. Results from ^{19}F NMR examination of acetonitrile extracts of exposed plants demonstrated that multiple

metabolites containing the parent compound were present internal to plants and metabolites and parent material internal to plants were quantified. Finally, fate of plant-sequestered contaminants in an anaerobic bioassay was examined using *Desulfitobacterium* sp. strain Viet1. Results indicated that the fraction of contaminant that was internal to plants, but not enzymatically processed by plants was readily released into aqueous phase in active and inactive systems. In addition, a portion of the contaminant that had been enzymatically processed by plants was available for release into aqueous phase and reduction by strain Viet1. A significant portion of contaminant sequestered by plants was not readily available in the strain Viet1 dominated system, indicating that a portion of contaminant sequestered by plants was not readily available to biological degradation.

The results of this study address the role of aquatic plants in sequestration of contaminants in surface waters that indicate the potential and limitations of use of aquatic plants in natural and engineered treatment systems.

CHAPTER 1

INTRODUCTION

Investigation of effects of chemicals on the environment increased in the late 1960's because of heightened public awareness of ecotoxicity, driven by Rachel Carson's documentation of ecological damage due to pesticide applications in *Silent Spring* (1962). Carson (1962) focused attention on the interconnected web of life by recounting how DDT residues could be found in deep-sea squid, Antarctic penguins and the fatty tissues of humans. DDT exposure was linked not only to avian infertility, but it was also one of the first cases associating cancer with a particular chemical (LaGrega *et al.,* 1994). This expose by Carson (1962) brought attention to the concept of biomagnification of toxins, where humans and other predators are most drastically affected. This, among many others, initiated the focused attention that has been given to food-crop accumulation of toxins, in particular heavy metals, herbicides and pesticides. In addition to concerns of bioconcentration of toxins, more recent attention has also been given to formation of metabolic products stemming from organic toxins which have the potential of being more, or less, toxic than the parent compound.

Stemming from observations that plants can sequester and transform toxins, scientists have looked at harnessing the detoxification powers of plants in an effort to

decontaminate soil and water. The field of phytoremediation has blossomed in the wake of related discoveries, with many efforts made to elucidate the fate of organic contaminants in plant systems. Much of the work completed to date has involved the use of trees (e.g., poplar and willow) due to their extensive root system and vast water uptake and grasses (rye and alfalfa) due to their extensive ground coverage and rhizoshpere structure. However, few studies have investigated the fate of organic pollutants in wetland or submerged aquatic plant systems. The movement of organic contaminants through groundwater aquifers, into low-lying seepline areas and into sediments of surface-water streams is well documented, as is extensive contamination of surface waters and wetland systems through direct application. These facts necessitate the investigation of the fate of organic contaminants in wetland and surface water systems. A fundamental understanding of the roles plant systems have in remediation and attenuation of organic contaminants in these systems is needed.

Problem Statement

While several studies have been completed on use of terrestrial plants to remove organic pollutants from soil or groundwater, few studies have been completed on the use of aquatic plants to remove organic pollutants from surface waters. Currently there is also a lack of understanding regarding how plant metabolic activity and health affects plant pollutant transformation. In a recent review, Trapp and Karlson (2001) mentioned the need for such studies by stating "Some questions are not yet solved, e.g. the optimal dosage (avoiding phytotoxicity)." Elucidation of the effects of pollutant transformation

on plant metabolic activity will allow sustainable system development of engineered wetlands and natural attenuation systems.

Chlorinated phenols are a USEPA priority pollutant found in surface waters, wetland systems, soil and groundwater. Chlorinated phenols have been selected as model organic contaminants for this study.

Objectives

The objective of this research was to explore fate of organic contaminants in aquatic plant systems through i) experimental development of relationships to describe sorption, uptake and enzymatic processing of contaminants by plants and inhibition of aquatic plants by contaminants and ii) incorporation of experimental relationships into a conceptual model which describes contaminant fate in aquatic plant systems. The specific objectives of the dissertation are described below.

1. Development of an assay to quantify plant metabolic activity and inhibition of aquatic plants by organic contaminants.

2. Establish relationships for fundamental interactions between plants and contaminants that dictate uptake, enzymatic processing and sequestration of contaminants by aquatic plants.

3. Delineate the primary chemical and physical properties of contaminants affecting uptake and enzymatic processing of organic contaminants by plants.

4. Establish ^{19}F NMR as a technique to quantify transformation and conjugation products internal to plants and contaminant assimilation by plants.

5. Develop, parameterize and independently verify a conceptual model which incorporates plant activity and inhibition in a mathematical description of uptake of organic contaminants by aquatic plants.

6. Examine availability of plant-sequestered contaminants after plant inactivation in anaerobic microbial systems.

These objectives were met through collaborative research which combined fundamental principles with advanced analytical techniques. This research represents an interdisciplinary research effort in environmental engineering which draws on strengths in environmental and analytical chemistry, aquatic toxicology, microbiology and physico-chemical processes.

CHAPTER 2

LITERATURE REVIEW

This review begins with a description of aquatic plants used for experimentation included herein. It continues with a discussion of work describing uptake of organic contaminants by aquatic plants and sorption of organic contaminants to aquatic plants. Next, a description of the model provided by Trapp and McFarlane (1995) which outlines enzymatic processing of organic contaminants by plants is followed by a description of current research which has elucidated transformation products of organic contaminants in aquatic plant systems. Studies where inhibition was observed in investigation of enzymatic processing of organic contaminants by aquatic plants are described. Toxicity assessment is then discussed, with emphasis on organism and cellular level toxicity indicators. Next, a discussion of the chlorophenols, the model contaminant class used, is provided with an emphasis on 2,4,5-trichlorophenol (2,4,5-TCP). This discussion includes chlorinated phenol uses, properties, toxicity and degradation routes and compares fluorinated analogs with their chlorinated phenol counterparts. Finally, a description of nuclear magnetic resonance (NMR) is given, with an extended description of ^{19}F-NMR applications.

Description of Aquatic Plants

Descriptions of the plants used, according to Westerdahl and Getsinger (1988), are summarized below. *Lemna minor* is a small floating plant that contains flat, 2-5 mm, nearly symmetrical, green fronds (leaves). Fronds may occur singly, or grouped into colonies of 2-4 fronds. *Lemna minor* is indigenous to many freshwater habitats throughout the world. The primary mode of reproduction is asexual budding. *Myriophyllum aquaticum* is a submersed/immersed perennial with typically unbranched stems that grow to 1.5 m tall. The foliage may extend up to 20 cm above the surface and is typically bright green. The plant is commonly found in lakes, ponds and canals and it spreads by fragmentation. *Elodea* spp. are submersed, rooted, perennial aquatic macrophytes found in quiet water or slow moving streams. The plant has branched stems arising from root crowns with 4-6 leaves in whorls that are 25 mm long and 2-5 mm broad. *Elodea* plants can form very dense mats. This plant is native to South America, but it has been introduced to waters throughout the world. *Ceratophyllum demersum* is a submersed perennial with highly branched stems and no roots. Leaves form in groups of 5-8 along the stem and grow up to 3 cm in length. *Ceratophyllum demersum* is one of the most abundant freshwater aquatic plants. It is usually found in standing water and forms dense colonies.

It is widely known that plant systems always exist symbiotically with microorganisms (McCutheson and Schnoor, 2003). In one common use of this symbiotic relationship, microbial bioremediation can be coupled with phytoremediation by using select vegetation to stimulate root associated bacteria (Olson *et al.,* 2003). In addition, fungi

exist either in free-living saprophytic form or in symbiotic association with vegetation and plant roots and are considered to be important degraders in the environment. Therefore, aquatic plants live as a part of a symbiotic consortium which includes bacteria, fungi and algae. Degradation or sequestration processes attributable to plant systems can be separated from those mediated by microorganisms using cell culture, axenic plants and appropriate controls, however applicability of cell culture results to full scale systems and the difficulty in excluding bacteria from cells have been questioned by researchers (McCutheson and Schnoor, 2003). Therefore, it is important to consider total contaminant flux in plant-microbe consortia relative to total biomass of each organism groups present, as well as endpoints for degradation, conjugation and sequestration processes when assessing contributions from plants and microbes.

Uptake, Sorption, and Enzymatic Processing of Organic Contaminants in Aquatic Plant Systems

Terrestrial plants such as trees and grasses have been established as an effective means of volatilizing or transforming organic contaminants. Hybrid poplar trees have been shown to volatilize or transform numerous organic environmental hazards, with recent efforts summarized in Aitchison *et al.* (2000). A recent study showed ~82% volatilization, mineralization, transformation or sequestration of 1,4-dioxane by hybrid poplars (Aitchison *et al.,* 2000). Schnabel *et al.* (1997) found that carrots, spinach and tomatoes sequestered, transformed or volatilized 74-95% of the applied trichloroethene. Burken and Schnoor (1997) found that hybrid poplars can readily uptake, hydrolyze, and dealkylate atrazine to less toxic metabolites. After 80 days, plants grown in soil had

assimilated 29.2% of the applied atrazine, while plants grown in sand assimilated 71% of the atrazine after 14 days. In the 80-day test, 15.8% was non-extractable bound residue, with the remaining material consisting of atrazine or its metabolites found in the roots and leaves. Degradation products identified were hydroxyatrazine, deethylatrazine, deisopropylatrazine, deethylhydroxyatrazine, and didealkylatedatrazine. A compartmentalized model was proposed for processes involved in metabolism of atrazine.

While several studies have been completed on use of terrestrial plants to remove organic pollutants from soil or groundwater, few studies have been completed on the use of aquatic plants to remove organic pollutants from surface waters. Uptake of organic contaminants, sorption to plants and enzymatic processing of organic contaminants has been investigated in aquatic plant systems. A heavy emphasis has been placed on understanding enzymatic processing and sequestration of contaminants, and some related studies have investigated kinetics of contaminant uptake or plant stress associated with contaminant uptake.

Uptake of Organic Contaminants by Aquatic Plants

Rate of contaminant uptake by aquatic plants has been studied using plants such as *Myriophyllum aquaticum*, *Spriodela oligorrhiza*, *Elodea canadensis*, *Ceratopyllum demersum*, *Lemna minor*, *Myriophyllum spicatum*, *Bacopa caroliniana*, *Elodea densa*, *Eichhornia crassipes* and *Lemna gibba*. A number of contaminants have been examined in aquatic plant systems including pesticides, nitroaromatics, and chlorinated phenols,

and several models have been provided to describe uptake and bioconcentration of organic contaminants by aquatic plants. Finally, relationships have been developed that can be used to predict uptake of various organic contaminants in terrestrial plant systems (Briggs *et al.,* 1987; Burken and Schnoor, 1998).

Uptake of Pesticides by Aquatic Plant Systems. Several studies have examined the ability of aquatic plants to detoxify herbicides and pesticides because runoff/erosion from agricultural fields is a large contributor to water quality degradation. Herbicides and pesticides can be relatively water soluble, adding to their frequency of detection in aquatic environments. The non-target effect of agricultural runoff into aquatic systems can be of great concerns for humans due to long-term exposure to low concentrations of contaminants in drinking water. Additionally, insecticides significantly impact aquatic arthropod populations and herbicides suppress the growth of aquatic vegetation (Leonard, 1990).

The uptake of organophosphorus pesticides (malathion, demeton-S-methyl, and crufomate) was investigated using axenically cultivated *Myriophyllum aquaticum, S. oligorrhiza* L., and *Elodea canadensis* (Gao *et al.,* 2000; Gao *et al.,* 2000b). Batch uptake studies performed over 8 days showed that *Myriophyllum aquaticum* took up 83%, 78% and 58% of malathion, demeton-S-methyl and crufomate, respectively, and dual-mode kinetics were observed. An initial rapid decrease in aqueous concentration was observed (i.e., 8.6% of malathion disappeared from *Myriophyllum aquaticum* culture medium within 16 hr of incubation) followed by a gradual, first-order concentration

decline in culture medium (i.e., 83% removal by *Myriophyllum aquaticum* after 8 d). Parallel initial contaminant losses occurred in autoclaved plant control systems, followed by contaminant concentrations reaching plateau values. Initial contaminant losses observed in active and inactivated plant systems were attributed to contaminant sorption to plant surfaces. Similar trends in uptake kinetics were observed for demeton-s-methyl and crufomate, where 2.1% and 1.8% of contaminant were removed by 16 hr and 78% and 58% of contaminant were removed by 8 d. However, uptake kinetics observed for *S. oligorrhiza* systems included a lag phase of 2.5 d and 3.5 d for demeton-S-methyl and crufomate, after which contaminant uptake proceeded at a rapid, first order rate. Finally, *Elodea canadensis* exhibited a lag-phase for uptake of malathion, extremely rapid uptake of demeton-S-methyl and no significant uptake of crufomate. Uptake rates and half lives of pesticides in plant systems depended on characteristics of both the plant and the pesticide. There was no indication that a specific plant was well suited for uptake of all pesticides and no pesticide studied was readily taken up by all plants examined. No relationships existed between physicochemical parameters of pesticides and mass of pesticide taken up by plants.

Aquatic plants, *C. demersum* L. and *Elodea canadensis*, were observed to accelerate removal and biotransformation of two herbicides, metolachlor and atrazine from contaminated waters (Rice *et al.,* 1997). In 16 days, both plants took up > 90% of the metolachlor and a significant amount of atrazine. *Lemna minor* was also tested for uptake abilities and was observed to remove a significant portion of the metolachlor, but did not have a significant effect on the concentration of atrazine. Half lives of

metolachlor and atrazine in vegetated systems were 8 d^{-1} and 78 d^{-1} (*Lemna minor*), 3d^{-1} and 25 d^{-1} (*Elodea canadensis*) and 3 d^{-1} and 12 d^{-1} (*C. demersum*) indicating that metolachlor was taken up more rapidly than atrazine by the plants examined. Lockhart and co-workers (1981) noted that a series of pesticides (fenitrothion, t-permethrin, ethalfluralin, krenite, terbutryn, dichlorodiphenyltrichloroethane (DDT), 2,2,,4,4,5,5-hexachlorobiphenyl and fluridone) accumulated in *Lemna minor* at various bioconcentrations (Bioconcentration Factor = 2.1 – 3671 after 96 hr).

Uptake of contaminants by aquatic plants was modeled by Wolf and co-workers (1991) where plants *Myriophyllum spicatum*, *H. verticillata* and *B. caroliniana* and contaminants 1,2,3,4-tetrachlorobenzene and 1,2,-dichlorobenzene were used to parameterize the model. Uptake, sorption and bioconcentration by submerged plants were examined through comparison between active and inactive plants. Contaminant accumulation results were similar for active and inactivated shoots for the first 3 hr of exposure, after which the inactivated shoots accumulated significantly more contaminant than the fresh shoots. Dynamics of diffusive transfer of chemical between aqueous solution and plant shoots and expected bioconcentration were explored by separately parameterizing processes occurring in leaves, stems and aqueous medium. Results presented indicated that other processes such as contaminant metabolism were occurring in active plant systems, however these processes were not included in the model. Gobas and co-workers (1991) examined partitioning between aqueous and plant phases for tri- to hexa-chlorinated benzenes and biphenyls (log K_{ow} values: 4.02 – 8.26) with low initial concentrations ranging from 0.072 – 5.92 μg/L. A flow-through system was used with

the aquatic macrophyte *Myriophyllum spicatum* where contaminant uptake over 25 d and contaminant elimination over 133 d were monitored. Contaminant transformation by plants was not observed or examined.

Uptake of Nitroaromatics by Aquatic Plant Systems. The fate of 2,4,6-trinitrotoluene (TNT) has been evaluated in aquatic plant systems. Axenic *Myriophyllum aquaticum* was observed to remove substantial amounts of TNT from an aqueous system (Hughes *et al.,* 1997). Batch uptake studies demonstrated complete removal of TNT from aqueous medium by *Myriophyllum aquaticum* after 5 d. An initial rapid decrease in aqueous concentration was observed (i.e., 22% of TNT disappeared from *Myriophyllum aquaticum* culture medium within 24 hr of incubation) followed by a gradual, first-order concentration decline in culture medium (i.e., complete removal of TNT by *Myriophyllum aquaticum* after 168 hr). Parallel initial contaminant losses occurred in heat inactivated plant control systems, followed by contaminant concentrations reaching plateau values. Kinetics of initial time points for aqueous TNT concentrations were paralleled by those of ^{14}C-TNT and initial contaminant losses observed in active and inactivated plant systems can be attributed to contaminant sorption to plant surfaces. ^{14}C-TNT measurements did not parallel aqueous TNT concentration measurements after the first sampling time point. Significantly more ^{14}C material remained in aqueous phase at later time points, where only 55% of ^{14}C material had been removed from the aqueous phase after 168 hr, whereas complete removal of aqueous TNT was observed by 168 hr. These results demonstrated that a significant portion of TNT or its derivatives

accumulated in plant material, and that metabolic products of TNT were released into aqueous phase.

Pavlostathis and co-workers (1998) examined kinetics of uptake of TNT by *Myriophyllum aquaticum* at a number of contaminant concentrations. Uptake of TNT by *Myriophyllum aquaticum* was modeled as a mixed, second order rate which was described as function of mass of plant inoculated and followed a trend related to initial TNT concentration. Sorption of TNT to plant surfaces was examined using azide inactivated controls, and sorptive processes similar to those noted by Hughes and co-workers (1997) were observed. Research examining TNT uptake by *Elodea densa* demonstrated similar rapid initial removal in active and inactive systems (Gueriguian, 1996). TNT concentrations reached a plateau concentration after an initial rapid decrease in aqueous concentration in inactivated plant systems. First order contaminant uptake proceeded after initial rapid sorptive processes in active systems (Gueriguian, 1996). Uptake of TNT by *Elodea densa* was modeled as a mixed, second order function of plant mass. Gueriguian (1996) also presented results for Salvinia, Iris, Pickerel weed and water chestnut, and similar kinetic analyses were assigned.

Model of Uptake Kinetics of Technetium-99 by Lemna minor. Uptake of the radionuclide technetium-99 (^{99}Te) by *Lemna minor* was modeled using a two compartment model (Hattnik *et al.,* 2000). Results showed an active uptake mechanism for accumulation of ^{99}Te in plants and followed by light induced reduction of contaminant internal to plants.

Uptake rate was first order and depended linearly on temperature, but did not depend on light input. No sorption to plant surfaces was apparent or noted.

Uptake of Chlorinated Aliphatics by Aquatic Plants. Hexachloroethane (HCA) and tetrachloroethene (PCE) were observed to be rapidly removed from aqueous phase in batch reactors by *Myriophyllum aquaticum* and *Elodea canadensis* (Nzengung *et al.,* 2003). Data presented by Nzengung and co-workers indicated >50% removal in less than 1 hr. The authors suggest that rapid initial removal could be attributed to sorptive processes which were explored in autoclaved plant controls. Further contaminant removal from the aqueous phase proceeded at an apparent first-order rate, however the authors did not consider an uptake step, but described a slow phytotransformation as the process which followed sorption. Contaminant removal was modeled using two pseudo first-order equations where the first described sorption and the second described phytotransformation. No control data were provided for comparison.

Uptake of Chlorinated Phenols by Plants. Interactions of chlorinated phenols with many different terrestrial and aquatic plant systems have been examined with efforts focused on understanding long term fate of chlorinated phenols and transformation products produced by plants. Few efforts have focused on understanding kinetics of chlorinated phenol uptake by plants. In a typical example of examination of chlorinated phenols in terrestrial plant systems, the fate of radiolabelled 2,4,6-trichlorophenol was explored in hydroponic tomato plants (Fragiadakis *et al.,* 1981). Using a ^{14}C-label experimentation, 20.0% of the 2,4,6-trichlorophenol was attributed to the roots and 1.2% to the stems and

leaves over a period of 30 days. No further kinetic analyses were performed in these systems.

The aquatic plant, *Eichhornia crassipes* was used to study uptake and elimination kinetics and metabolism of pentachlorophenol (Roy and Hanninen, 1994). It was observed that pentachlorophenol concentration dropped sharply after the first 12 hours of exposure, and the rate of pentachlorophenol disappearance quickly decreased, reaching a steady state concentration by 48 hours. Elimination kinetics were then observed by placing plants which had accumulated pentachlorophenol in clean media and observing loss of material from the plant over time. Pentachlorophenol release was observed to be slower than uptake. Roy and Hanninen (1994) stated that a plant-water, two-compartment model was insufficient to explain kinetics observed, and assumed a multi-component model wherein the plant was divided into separate compartments (leaf, stem, root). This model is in agreement with other studies that used complex formulas to estimate bioconcentration and partitioning properties of organic chemicals (Wolf *et al.,* 1991; Gobas *et al.,* 1991).

Ensley and co-workers (1994) provided evidence for first-order removal of 2,4-dichlorophenol (2,4-DCP) from aqueous phase by *Lemna gibba* as determined by GC-MS measurements. A parallel study was performed using ^{14}C-2,4-DCP where removal of ^{14}C material was slower than 2,4-DCP losses from media as measured by GC-MS. Therefore, significantly more ^{14}C material remained in aqueous phase after 6 d than 2,4-DCP detected by GC-MS. However, the authors showed results for GC-MS analyses in

units of peak area, not concentration, therefore calculations for total removal from reactors using GC data were impossible. Evidence of polar metabolites in aqueous phase was claimed, however descriptions of analytical techniques involved homogenizing plant and aqueous phase into one slurry for extraction and subsequent detection of metabolites, thus negating any evidence of metabolic products released into aqueous phase. Further, results presented by Day (2002) and those presented in later sections of this document were not consistent with incomplete removal from the aqueous phase or any net accumulation of products in aqueous phase.

Predictive Relationships to Describe Uptake in Terrestrial Plant Systems. Relationships have been developed to predict contaminant uptake by terrestrial plants where partitioning processes were related to the log of the octanol-water partition coefficient (log K_{ow}). Briggs and co-workers (1982) gauged contaminant uptake by barley using root concentration factor (RCF) and transpiration stream concentration factor (TSCF). RCF was defined as the concentration of contaminant in the roots normalized to the concentration in external solution, while TSCF was the concentration in the transpiration stream normalized concentration in external solution (Briggs *et al.,* 1982; Burken and Schnoor, 1998). Both ratios were independent of time and concentration. Values for RCF and TSCF were correlated to log K_{ow} of the herbicides and then used to predict herbicide uptake by plants. Corrections for degradation were incorporated in TSCF by assuming first-order metabolism of contaminants by plants; however, these corrections were considered small by Briggs and co-workers (1982) and were only applied in a fraction of contaminants tested. Subsequent work by Burken and Schnoor (1998)

examined uptake of a variety of priority organic chemicals that held potential for phytoremediation with hybrid poplar trees. TSCF was used to characterize contaminant removal from batch reactors and was limited by root partitioning as represented by RCF. Briggs and co-workers (1982) and Burken and Schnoor (1998) identified correlations between TSCF values and log K_{ow}. Gaussian curves correlated TSCF with log K_{ow} with optimum TSCF values observed at log K_{ow} of 1.78 and 2.50, respectively. Contaminant transformation was not modeled as a major removal process in the preceding predictive relationships.

Enzymatic Processing of Organic Contaminants by Aquatic Plants

Enzymatic processing of organics has been studied in aquatic plant systems. A model which provides an overview of organic processing by plants is presented along with evidence for transformation, conjugation and sequestration of pesticides, TNT and chlorinated phenols by aquatic plants. In addition, a brief description of metabolism of chlorinated phenols by microorganisms is provided and compared with typical metabolic pathways occurring in aquatic plant systems.

Transformation Processes Involved in Enzymatic Processing by Plants. Often called the "green liver" of the earth, plant metabolism of contaminants can be treated conceptually like liver metabolism (Trapp and McFarlane, 1995). This concept divides contaminant processing into three conceptual phases similar to the way liver metabolism is depicted (Figure 2.1). This model has been used in this research as a general guide for the fate of contaminants within plants.

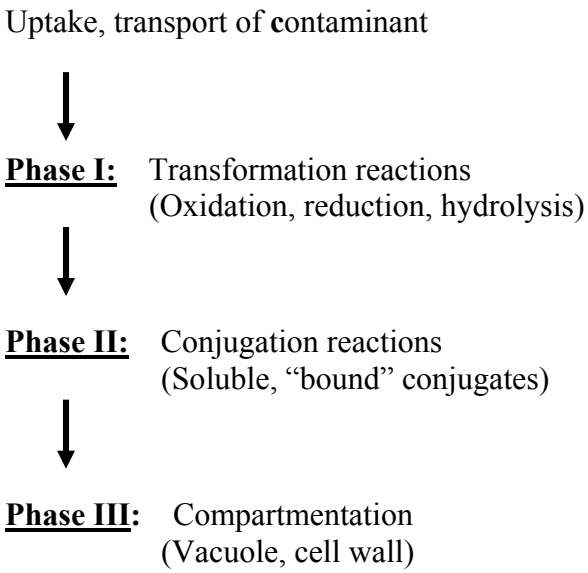


Figure 2.1. Plant metabolism of contaminants after Trapp and McFarlane (1995).

Phase I involves transformation reactions where a plant typically introduces functional groups (*e.g.,* -OH, -NH_2, -SH) into a contaminant compound. Oxidation via aromatic or alkyl hydroxylation, nitrogen or sulfur oxidation, epoxidation and O- or N-dealkylation are frequently observed. These oxidation reactions often lead to detoxification or activation of a contaminant to facilitate further contaminant metabolism. Reductive reactions have been found for certain nitroaromatic compounds, and hydrolytic reactions may occur with carboxylic acid esters, organophosphorus compounds, carbamates and anilides.

Phase II consists of formation of soluble or insoluble ("bound") conjugates. The main reaction types for soluble conjugates include glucoside, glutathione, amino acid, and malonyl conjugation where substituents are attached to functional groups existing on the parent compound or incorporated into the parent compound in Phase I. Phenols and alcohols are commonly conjugated as β-O-glucosides, and those with an amino group are metabolized to N-glucoside. Glucose ester conjugates are formed with compounds containing carboxyl groups, while glutathione conjugation typically involves displacement of a halogen or nitro group. Compounds can also be incorporated into complex biopolymers that are insoluble to commonly used solvents.

Formation of glucoside conjugates is catalyzed by glycosyltransferase enzymes. Formation of glucoside conjugates is a major detoxification mechanisms used by plants and animals including humans, therefore the broad class of glycosyltransferase enzymes has been examined extensively for the purpose of furthering the medical practice (Meech

and Mackenzie, 1997; Gueraud and Paris; 1998; Bossuyt and Blanckaert, 2001; Meech and Mackenzie, 1998). Glycosyltransferase have been shown to be bound to the endoplasmic reticulum in animal cells (Meech and Mackenzie, 1997; Gueraud and Paris; 1998; Bossuyt and Blanckaert, 2001). Meech and co-workers (1997) demonstrated that substrate binding for gluconyltransferase occurs within the endoplasmic reticulum in rat cells. In plant systems, glycosyltransferase enzymes have been described as soluble or membrane-bound enzymes (e.g., golgi bodies and endoplasmic reticulum) (e.g., Keegstra and Raikhel, 2001; Couthinho *et al.,* 2003).

Experimental work by Soffers and co-workers (1994) examined kinetics of rat gluconyltransferase enzymes with various halogenated phenols. Effects of structural and electronic characteristics on the Michaelis-Menton half velocity constant (K_m) of glucuronyltransferase were monitored in rat cell microsomes with various halogenated phenols with a changing halogen substituent on 2-fluoro-4-halophenols (Soffers *et al.,* 1994). No correlation was observed between structural and electronic characteristics that focused on the oxygen moiety in the phenol (e.g., highest occupied molecular orbital density on oxygen, net charge on oxygen) and measured K_m values. These results led the authors to conclude that contaminant hydrophobicity was the parameter that varied with substituent due to increased accumulation of the phenol in the membrane environment of the UDP-glucuronyltransferase enzymes. However, contaminant pK_a and speciation in cells were not examined as factors influencing contaminant conjugation.

In Phase III, many aromatic compounds with existing or introduced hydroxyl, carboxyl, amino, or sulfhydryl groups are deposited into lignin or cell wall components, while other components are known to be deposited in a vacuole. Reactions of Phase II and III may occur simultaneously or separately.

An examples of plant metabolism of contaminants that fit to the "green liver" metabolism of contaminants is provided in Figure 2.2. These enzyme-mediated reactions with pentachlorophenol are not yet completely documented as being plant mediated, but are shown to provide an example from many possible scenarios (Trapp and McFarlane, 1995).

In an effort to delineate reactions involved in a similar model, several studies have examined sequestration and reduction of heavy metals by terrestrial and aquatic plants. The treatment of organics has been less thoroughly investigated. Many studies have investigated enzymatic processing of organic contaminants by aquatic plants through identification of transformation products and through investigation of contaminant which was bound in a non-extractable form to plant materials. Bound residues are non-extractable and therefore defy common analyses. The definition of bound residues has typically varied between scientist and extraction procedure used. IUPAC has set forth a series of guidelines for defining bound residues as those non-extractable residues of chemical species originating from pesticides which are not extracted by methods which exhaustively remove chemical species from a plant matrix but do not significantly change the chemical nature of these residues (Skidmore *et al.,* 1988). Bound residues exclude

Phase I **Phase II** **Phase III**

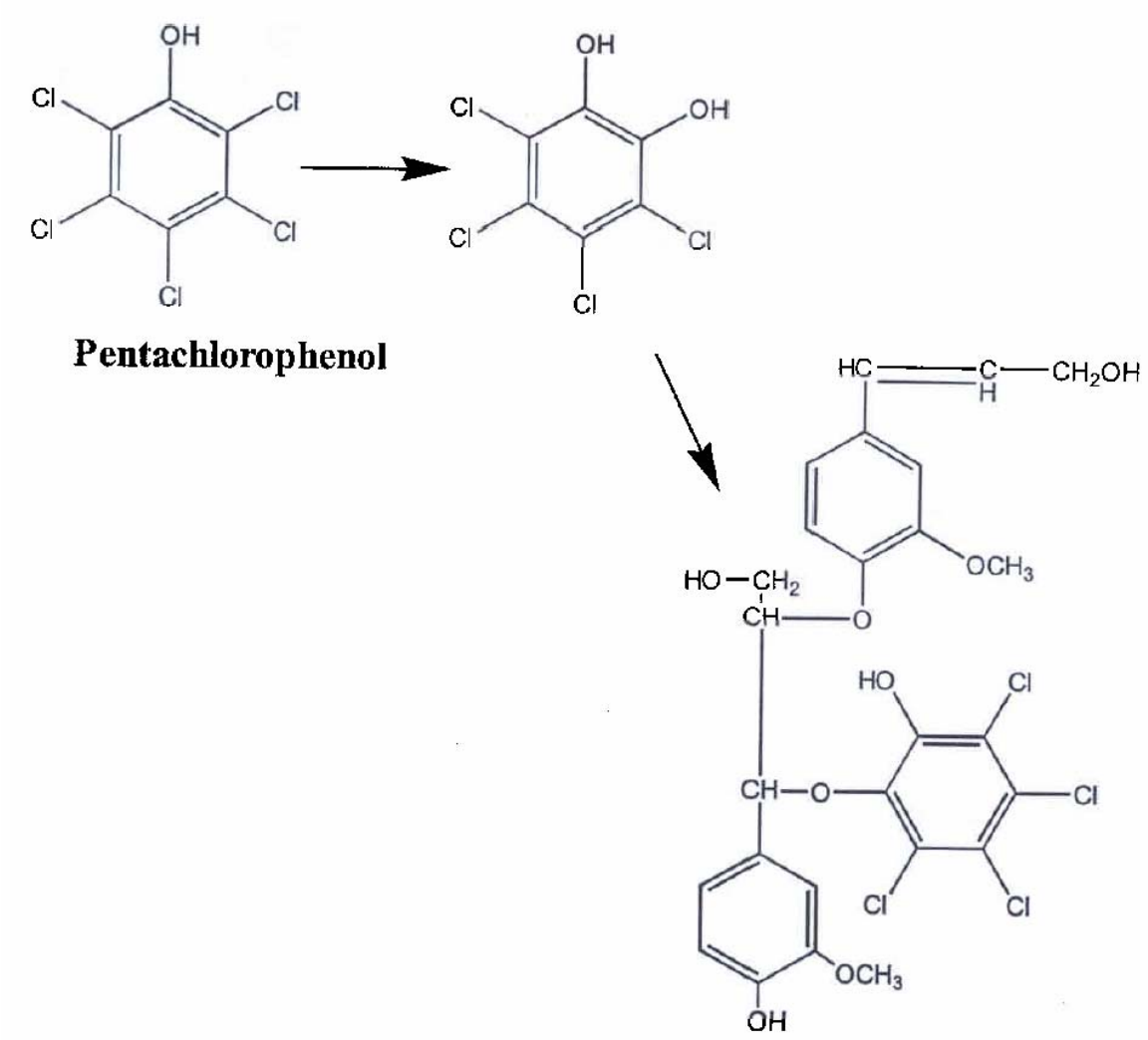


Figure 2.2. Possible reaction sequence of pentachlorophenol in plant tissue after Trapp and McFarlane (1995).

fragments which are recycled through pathways lead into natural incorporation. Bound residue are typically detected using radioactive labeling, although recent efforts have investigated use of stable carbon labeling for bound residues identification (Richnow, 1999).

Transformation of Pesticide Compounds by Aquatic Plants. Studies performed by Gao and co-workers (2000) examined transformation of malathion, demeton-S-methyl and crufomate by *Myriophyllum aquaticum*, *S. oligorrhiza* and *Elodea canadensis*. A portion of each pesticide studied accumulated in plants and experimentation with ^{14}C labels demonstrated that 29%, 82% and 17% of the malathion, demeton-S-methyl and crufomate assimilated by *Myriophyllum aquaticum* was bound in a non-extractable manner (Gao *et al.,* 2000, Gao *et al.,* 2000b). Although no mass balance was performed, the involvement of enzymatic reactions in live plant systems was evidenced by the fact that material adsorbed by autoclaved controls was readily extracted, whereas plant extracts of live plant systems did not readily provide the parent compound (Gao *et al.,* 2000b). The small amount of material taken up by killed controls did not undergo any enzymatic reactions; thus tissue extractions readily produced the parent compound. The fact that material taken up by inactivated plants was readily extractable further confirms conclusions regarding sorptive processes occurring in inactivated systems. Tissue extracts confirmed transformation abilities of the three plants for the three herbicides tested and provided data that fit into the "green liver" metabolism of contaminants concept, where Phase III reactions formed the non-extractable, bound material.

S. oligorrhiza cell extracts demonstrated enzymatic transformation of the herbicides. This portion of the study showed faster transformation of all three herbicides, as there was no limitation from transport of material into the plant cells, however the enzyme extracts did not transform all material available. While metabolic products were not identified, transformation was attributed to the activity of an organophosphorus hydrolase. This is a well-characterized enzyme that has the ability to hydrolyze a variety of organophosphorus compounds with P-O bonds as well as P-S bonds (Gao *et al.,* 2000b).

A related study observed the fate of the organophosphorus pesticide chlorpyrifos in a model ecosystem dominated by the aquatic macrophyte, *Elodea nuttallii* (Brock *et al.,* 1992). The concentration of chlorpyrifos extracted from *Elodea nuttallii* significantly decreased through the experiment, with tissue extractions providing 17.4 μg chlorpyrifos per g dry weight at 8 days and 4.6 μg chlorpyrifos per g dry weight at 30 days. It was suggested that removal of chlorpyrifos was due to external sorption and subsequent hydrolysis or photolysis. Previous work by Macalady and Wolf (1983) suggested that alkaline hydrolysis could be an important factor as the rate of hydrolysis of chlorpyrifos is particularly high under alkaline conditions and dense beds of aquatic macrophytes may cause the pH of ambient water to reach high alkaline levels.

The persistent, organochloride pesticide, DDT, has also been investigated for degradation by axenically cultivated *Myriophyllum aquaticum, S. oligorrhiza*, and *Elodea canadensis* (Gao *et al.,* 2000). This study observed accumulation and degradation of enantiomers o,

p'-DDT and p,p'-DDT. In *Myriophyllum aquaticum* exposure, both enantiomers disappeared rapidly during the first 16 hr of exposure, followed by a decrease in uptake rate. After 6 days, neither enantiomer could be detected in the culture medium, but parent compounds o,p'-DDT and p,p'-DDT and metabolites o,p'-DDE, p,p'-DDE, o,p'-DDD, and p,p'-DDD, were detected in plants at 24, 18, 1.3, 1.9, 7.9 and 11.5%, respectively. *S. oligorrhiza* exhibited more extensive degradation, in which 66 to 50% of o,p'-DDT and p,p'-DDT were transformed. o,p'-DDD and p,p'-DDD are the major metabolites of duckweed exposure, and considerably more p,p'-DDE and less o,p'-DDD were produced by *S. oligorrhiza*. 31 and 48% of o,p'-DDT and p,p'-DDT respectively were bound by *Elodea canadensis.* A separate study found 22% removal of DDT by *Elodea canadensis*, but no degradation products were found (Garrison *et al.,* 2000). No degradation products were found in systems with autoclaved plants or unplanted systems. Gao *et al.* (2000) attributed metabolism of DDT enantiomers to dehalogenation and dehydrochlorination reactions involving a general plant dehalogenase. It can be conjectured that the transformation of DDT into DDE and DDD represents Phase I reactions and the fraction that was determined to be non-extractable was formed by a Phase III reaction, after the "green liver" concept. Garrison *et al.* (2000) used pure enantiomer observations to identify the enantioselectivity of the DDT transformation, concluding that disappearance of o,p'-DDT was not enantioselective, lending information about the enzymatic reaction producing the metabolites.

Degradation products DDD and DDE are also toxic and persistent in the environment, but Fox and co-workers (1998) indicated they can be further converted to polar, less toxic

compounds, such as 1-chloro-2,2-bis(p-chlorophenyl)ethylene (DDMU) and 2,2-bis(p-chlorophenyl)acetic acid (DDA) in rats.

Transformation of TNT by Aquatic Plants. Metabolic products of 2,4,6-trinitrotoluene (TNT) were evaluated using axenic *Myriophyllum aquaticum* and *Elodea densa* (Hughes *et al.,* 1997; Gueriguian, 1996; Bhadra *et al.,* 1999; Bhadra *et al.,* 1999; Vanderford *et al.,* 1997). In *Myriophyllum aquaticum* systems, metabolic products 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene were observed in both the plant tissue and the medium, and significant conversion of the material into a reduced state. No other metabolites were identified and mineralization was not observed. Heat-inactivated controls were noted to take up material over the first day, and then reach a constant value. In live systems, ^{14}C-label studies were performed to determine the fate of the compound. After 7 days, 50% of the ^{14}C remained in the aqueous medium, but only trace levels of TNT could be detected in the medium. The unidentified products did not match the elution profiles of other known nitroaromatics, and were thought to have limited UV/Vis adsorption and/or eluted in the polar solvent front (Hughes *et al.,* 1997).

A subsequent study examined the unidentified, oxidized and mostly acidic metabolic products produced by *Myriophyllum aquaticum* (Bhadra *et al.,* 1999). Compounds identified in the aqueous phase include 2-amino-4,6-dinitrobenzoic acid, 2,4-dinitro-6-hydroxy-benzyl alcohol, 2-N-acetoxyamino-4,6-dinitrobenzylaldehyde, 2,4-dinitro-6-hydroxytoluene and two binuclear metabolites different from typical azoxytetranitrotoluenes. These products demonstrated that *Myriophyllum aquaticum* has

the ability for oxidative transformations, methyl oxidation and/or aromatic hydroxylation. These oxidation reactions were indicated to precede the nitro-group reduction described previously, as none of the oxidative products were formed when 4-amino-2,6-dinitrotoluene or 2-amino-4,6-dinitrotoluene was the starting material for plant exposure. It is interesting to note that ring hydroxylation was observed with production of 2,4-dinitro-6-hydroxytoluene and 2,4-dinitro-6-hydroxy-benzyl alcohol, where hydroxylation is accompanied by the removal of a nitro group. These reactions fit into the "green liver" metabolism of contaminants, representing Phase I or Phase II reactions.

Transformation of Chlorinated Aliphatics by Aquatic Plants. Surface sterilized and gamma irradiated *Myriophyllum aquaticum* and *Elodea canadensis* plants were used to identify degradation products of hexachloroethane and tetrachloroethene (Ngeuzen *et al.,* 2003). No verification of microbial community status was provided and degradation products typical of microbial reduction of chlorinated aliphatics were offered as degradation products. Authors provided a short description of $^{14}C–CO_2$ captured from headspace without providing supporting evidence. Results presented require further investigation before claims of plant driven dechlorination processes and plant driven mineralization can be confirmed because data which substantiated claims of microbe-free systems, product identification and CO_2 evolution were not presented.

Transformation of Chlorinated Phenols by Plants. Long-term fate metabolic product formation from chlorophenols has been explored in plant systems. Several of these studies were performed to achieve a better understanding of bioconcentration effects in

edible plants, while others strive to establish plant degradation and detoxification patterns for the chlorophenol family in an effort to develop a remediation strategy. Table 2.1 summarizes the metabolic products found for chlorinated phenols.

Metabolism of chlorinated phenols was described to proceed primarily through glycosidation in *Lemna minor* (Day, 2002). Upon exposure of *Lemna minor* to 2,4-dichlorophenol (2,4-DCP), products of 2,4-dichlorophenyl-β-D-glucopyranoside (DCPG), 2,4-dichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside (DCPMG) and 2,4-dichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuanoside (DCPAG) were observed. Examination of 2,4-DCP metabolite production kinetics demonstrated that DCPG was the initial plant-metabolite and was the precursor for production of DCPMG and DCPAG. Parallel conjugation processes and patterns were identified for 2,4,5-TCP (Day, 2002). This study was consistent with previous reports of glycosidation as the initial step in chlorophenol metabolism by plants (*e.g.*, Ensley *et al.,* 1997). Formation of DCPG and DCPMG represent Phase II metabolism of 2,4-DCP and are considered to be components moved into vacuoles to minimize toxic effects. Apiose is a rare carbohydrate known to exist in *Lemna* sp. cell wall tissue, therefore formation of DCPAG is a Phase III metabolic process.

The identification of chlorophenol metabolites in aquatic plants has also been explored with the aquatic fern, *Lemna gibba.* Short-term exposure to 2,4,5-trichlorophenol, 2,4-dichlorophenol and phenol produced a β-glucoside conjugate as the major metabolic product (Sharma *et al.,* 1997; Ensley *et al.,* 1997; Ensley *et al.,* 1994; Barber *et al.,*

1995). A long-term study was completed with 2,4,5-trichlorophenol, which resulted in identification of several dechlorination products. Dichlorophenols, monochlorophenols and phenol were found both in plant material and re-released into the media (Ensley *et al.,* 1997). However, results indicating that metabolic products were released into the media or that dechlorination products were present were not substantiated by the work of Day (2002). Further, detection and identification of metabolic products was performed on a media and plant homogenate, thus eliminating the possibility of isolating products in the media.

The fate of 2,4,6-trichlorophenol was explored in hydroponic tomato plants (Fragiadakis *et al.,* 1981). Using a ^{14}C-label experimentation, 20.0% of the 2,4,6-trichlorophenol was attributed to the roots and 1.2% to the stems and leaves over a period of 30 days. This study identified several metabolic products, grouping them into categories of (i) lignin incorporated ether-like resides, (ii) cellulose incorporated residues, (iii) glycosidically bound detoxified residues, and (iv) non-extractable residues. Similarly, the fate of pentachlorophenol was explored in rice (*Oryza sativa* var. aristata) plants which were rooted in soil that was maintained under flooded conditions (Weiss *et al.,* 1982). This two-year study found that 12.9% of the contaminant was taken up in the first year and only 2.5% was taken up in the second year. These researchers identified several metabolic products of pentachlorophenol (Table 2.1) which were extracted from the roots, straw, husks and grain. Casterline *et al.* (1985) explored uptake and transformation of radiolabeled pentachlorophenol by soybean and spinach plants. After 46 days, the average radioactivity uptake per plant was 0.7% of the pentachlorophenol applied and

several dechlorination and transformation products were found in the soybean and spinach plants (Table 2.1).

Several metabolites of pentachlorophenol were identified by Roy and Hanninen (1994), as presented in Table 2.1. Many of the metabolites were bound or conjugated forms of pentachlorophenol, chloroanisole, chloromethoxybenzenes, chlorohydroquinones and catechols. In an effort to determine metabolite origin, researchers determined the activity of several enzymes involved in oxidative biotransformation and conjugation in aquatic plants including, peroxidase, ascorbate peroxidase, glutathione reductase, glutathione-S-transferase, superoxide dismutase, as well as total glutathione content. Metabolites observed were consistent with oxidative or conjugative functions of enzymes that measurably increased with contaminant exposure.

Metabolism of pentachlorophenol was also investigated in wheat, *Triticum aestivum* by Schafer and Sandermann (1988). After 48 hours, metabolites comprised 50% of the material exposed, with 31% being incorporated into an "insoluble" residue. A β-D-glucoside conjugate and tetrachlorocatechol were identified along with lignified components and tetrachlorohydroquinone. A metabolic pathway was proposed where pentachlorophenol was transformed into tetrachlorocatechol, which then becomes either O-malonyl-β-D-glucoside or a part of lignin or other biopolymers.

Pentachlorophenol degradation was also investigated in Hycrest Crested Wheatgrass (*Agropyron desertorum*) by Ferro *et al.* (1994). Findings included 22% mineralization in

20 weeks compared with 6% mineralization in unplanted systems. A total of 36% was found in plant tissue (21% root fraction, 15% shoot fraction). A greenhouse-scale experiment was performed to measure enhanced biodegradation of pentachlorophenol in the presence of *Lolium perene* (perennial ryegrass) by Ferro *et al.* (1997). At the 64-day sample, significant amounts of pentachlorophenol were removed from the planted and unplanted, nutrient amended systems while the unplanted, not-amended system showed little removal of pentachlorophenol. Analysis of plant shoot samples indicated that little pentachlorophenol remained in the plant shoots, and no detectable metabolites existed in the plant shoots.

Metabolites identified for pentachlorophenol readily fit into the model of plant-contaminant interactions outlined in the "green liver" concept. Chlorocatechols, chloroguiachols, and hydroquinone forms of pentachlorophenol identified by various studies are easily identified as being formed by Phase I. Lignin-bound and other non-extractable residues were found in every study performed, and are easily identified with Phase III reactions. Glucosides were also commonly identified metabolic products, fitting into Phase II. Lesser-chlorinated phenols were also observed, and were likely formed through a combination of Phase I and II reactions.

Metabolism of Chlorinated Phenols by Microorganisms. Pathways for metabolism of chlorinated phenols by microorganisms have been delineated and differ in many ways from those of plant systems. In aerobic systems, microorganisms typically proceed to introduce a hydroxyl moiety into the phenol ring through an oxygenase attack which

promotes chlorine removal, ring cleavage, and utilization of carbon associated with the chlorinated phenol (Rittman and McCarty, 2001). Chlorinated catechols, guiacols and resorcinols are the intermediate products generated in the process of microbial degradation of chlorinated phenols in aerobic systems. Reductive dehalogenation of chlorinated phenols has been observed in anaerobic systems, where lesser chlorinated phenols and phenol are primary degradation products (Rittman and McCarty, 2001). Conjugation and increasing contaminant complexity is not a characteristic pathway for microbial degradation of chlorinated phenols and products typical of plant conjugation and sequestration of chlorinated phenols have not been reported in microbial systems.

Effects of Inhibition on Aquatic Plant Uptake and Enzymatic Processing of Organic Contaminants

Aquatic plants are known to be inhibited by organic contaminants and are frequently used in toxicity assays to monitor toxicity of chemicals to aquatic environments as is detailed in subsequent sections. Assessment of toxicity in studies examining contaminant uptake and processing by plants has been explored in a small number of systems, although stress or inhibitory effects were likely affecting plant systems in many experimental systems published. Batch systems which exhibit reduced contaminant uptake (i.e., contaminant concentration reaching plateau values other than zero) could possibly have been inhibited by contaminants, but possibly not immediately at the whole plant level and therefore plants did not appear to be seriously affected by contaminants.

Stress triggered enzyme production was examined in *Eichhornia crassipes* plants during uptake, metabolism and elimination of pentachlorophenol (Roy and Hanninen, 1994). Pentachlorophenol concentration dropped sharply after the first 12 hours of exposure, and the rate of PCP disappearance quickly decreased, reaching a steady state concentration by 48 hr. Batch systems exhibited reduced contaminant uptake at later time points which can be considered an indicator of contaminant toxicity. Enzymes triggered by stress, such as peroxidase, glutathione and those in the Halliwell-Asada pathway, were observed to increase relative to non-contaminated controls during contaminant uptake. The increase in enzyme activity can be potentially linked to a decrease in chlorophenol uptake rates, indicating that the toxicant may have inhibited plant processes and potentially caused the decrease in uptake rates.

Plant inhibition was qualitatively evaluated in studies examining TNT-accumulation by *Myriophyllum aquaticum* by assessing chlorosis, leaf loss and lack of new growth as endpoints (Pavlostathsis *et al.,* 1998). Plants dosed with high concentrations of TNT (262 and 500 μM) showed chlorosis after 7 to 27 hr of incubation. Middle range TNT concentrations (49 and 127 μM) exhibited chlorosis after 123 hr of incubation. Plants exposed to low concentrations and non-contaminated controls did not show signs of chlorosis and exhibited new growth indicating that high TNT concentrations caused inhibitory effects in plants. Rate of contaminant removal from aqueous phase was shown to decrease with increasing concentrations, a possible effect of inhibition. Inhibition of *Elodea nuttalli* by TNT and effects on uptake rates was assessed by Benton (1997). High concentrations of TNT inhibited carbon fixation by plants as was assessed using

radiolabled bicarbonate and measuring ^{14}C loss from media over 4 hr. Carbon fixation rates were also shown to decrease with repeated exposure to lower concentrations of TNT. Kinetics of inhibition were modeled by incorporating an inhibition function into classical Michaelis-Menton type relationships with CO_2 as the substrate and TNT as the inhibitor. Rate of carbon fixation was a linear function of TNT concentration and an inhibition constant of 2.51 mg/L applied to experimental systems used.

Often, inhibitory effects occurred in experimental systems examining uptake of metabolism of organics by aquatic plants, but authors did not quantify inhibition or consider inhibition in data analyses. In one example, batch uptake studies performed by Gao and co-workers (2000) demonstrated that malathion, demeton-S-methyl and crufomate were rapidly removed from aqueous phase by *S. oligorrhiza.* However, a plateau concentration different from zero was reached for all pesticides examined. *Elodea canadensis* removed large percentages of malathion, but did not significantly remove crufomate. *Elodea canadensis* initially removed demeton-S-methyl more rapidly than the other two plants, with a drastic reduction in rate after 1 day. Crufomate uptake by *Myriophyllum aquaticum* reached a plateau concentration after 3 d of exposure, and after 3 d of plateau concentration, contaminant uptake continued at a first order rate. Crufomate reached a final plateau concentration after 11 d of exposure, and complete contaminant removal was not removed. No examination of relative toxicity of any of the pesticides examined to any plants examined was offered even though decreasing uptake rates can be indicative of inhibition. In another example, researchers studying uptake of PCE and HCA by *Myriophyllum aquaticum* and *Elodea canadensis* observed that plants

Table 2.1. Identified metabolic products of chlorinated phenols.

Citation	Compound	Plant	Metabolites
Day, 2002	2,4-DCP	*Lemna minor*	2,4-dichlorophenyl-β-D-glucopyranoside; 2,4-dichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside; 2,4-dichlorophenyl-β-D-
Day, 2002	2,4,5-TCP	*Lemna minor*	2,4,5-trichlorophenyl-β-D-glucopyranoside; 2,4,5-trichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside; 2,4,5-trichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuanoside
Ensley *et al.,* 1997; Sharma *et al.,* 1997	2,4,5-TCP	*Lemna gibba*	2,4,5-trichlorophenol-β-glucoside; phenol; 2-chlorophenol; 2,4-dichlorophenol; 3- and/or 4-chlorophenol; 3,4-dichlorophenol
Fragiadakis *et al.,* 1981	2,4,6-TCP	Tomato	Lignin/ether-like resides; cellulose residues; glycosidically bound residues; non-extractable residues
Weiss *et al.,* 1982	PCP	*Oryza sativa* var. aristata (Rice)	2,3,4,5-tetrachlorophenol; 3,4,5-trichloroanisole; 1,2 plus 1,4-dimethyloxytetrachlorobenzene; 2,3,4,5-tetrachloroanisole; pentachloroanisole; conjugated 3,4,5-trichloroanisole; 1,2-hydroxy and/or monohydroxy monomethosytetrachlorobenzene; 3,4,5-trichlorophenol;
Roy and Hanninen, 1994	PCP	*Eichhornia crassipes*	2,3-dichlorophenol; 2,6-dichlorophenol; 3,5-dichlorophenol; 4,5-dichloroguaiacol; 2,3,4-trichlorophenol; 2,3,6-trichlorophenol; 2,3,4,5-tetrachlorophenol; 2,3,4,6-tetrachlorophenol; 4,5-dichlorocatechol; 3,4,5-trichloroguiacol; 4,5,6-trichloroguaiacol; 3,4,5-trichlorohyroquinone; tetrachloroguiacol; tetrachlorohydroquinone; tetrachlorocatechol;
Ensley *et al.,* 1994; Barber *et al.,* 1995	2,4--DCP; ;Phenol	*Lemna gibba*	2,4-dichlorophenol-β-glucoside; Phenol-β-glucoside
Casterline *et al.,* 1985	PCP	Soybean, Spinach	2,3,4,6-tetrachlorophenol; pentachloroanisole; 2,3,4,6-tetrachloroanisole; methoxytetrachlorophenol

were stressed by exposure conditions, however plant stress was not quantified and was not accounted for in uptake modeling.

Plant Toxicity Responses

This section provides a general background for plant toxicity and health assessments. While the thrust of the research concentrated on health assessment of mature vascular aquatic plants, a brief introduction is provided to promote a general understanding of toxicity assessment. A thorough discussion of plant toxicity assessment, both terrestrial and aquatic, provides background and insight into choices made for plant health assessment performed in this research.

Toxicity Assessment Background

Toxicity assessment is used to identify environmental risk in many settings. Standard toxicity tests are used to identify contaminants of concern for human health, as well as assess risks to wildlife. Whole effluent testing (WET) in the wastewater reclamation profession uses standard toxicity testing techniques to determine the biological impact of releasing an industrial or municipal effluent into a receiving body (Maltby *et al.,* 2000). Complex effluents that are released into aquatic environments such as effluents from municipal and industrial facilities, including petroleum refineries, pulp and paper mills, wastewater treatment plants, and landfills, are commonly screened utilizing standard toxicity tests (Nwosu *et al.,* 1991; Bailey *et al.,* 2000; Sarakinos *et al.,* 2000).

WET is particularly useful for assessing unknown contaminants, complex mixtures, and hazardous wastes where a single component may injure only a single trophic level (Wang

and Freemark, 1995; Maltby *et al.,* 2000). The use of WET also removes the need to determine which component of a poorly characterized mixture is actually the toxic portion. WET also eliminates the need for chemical specific standards and the need to predict interactions between complex effluents (Maltby *et al.,* 2000). These screenings provide a relatively quick, sensitive, and cost-effective means of generating an initial assessment of potential environmental risks of effluent discharge, as well as provide guidance for development of environmental regulations and guidelines (Wang and Williams, 1988; Taraldson and Nordberg-King, 1990; Sherry *et al.,* 1997; Wang and Freemark, 1995).

The test species used in standard toxicity tests is a frequently-debated topic in the literature. Kenaga and Moolenaar (1979) compared toxicity assessment results of 49,000 chemicals among daphnids, fish, aquatic plants, and algae and concluded that environmental assessment based on toxicity tests for fish and daphnids is sufficiently restrictive to protect plants and algae. Wang and Williams (1988) questioned test methods used to reach these conclusions, and pronounced that sensitivity of a test organism to toxic substances is a complex issue, involving types of toxicants, environmental conditions, test methods and many other factors. Researchers have subsequently shown that sensitivity of plants and other groups of organisms varies widely among toxicants. For example, results show that the decreasing order of organism sensitivity to Cd, Cu, and Zn followed: algae > daphnids > bacteria > plant seeds > earthworms and the decreasing order of sensitivity to herbicides followed: plant seeds >> algae = bacteria > daphnids >> earthworms (Wang and Freemark, 1995). Wang and

Williams (1988) indicated that toxicity of many herbicides is often 1000 times higher to plants than many other commonly used faunal species. Many other hazards such as detergent surfactants, textile effluents, acridine, dyes, and synthetic fuels are considered to be notably more toxic to algae and plants than to invertebrates and fish (Geis *et al.,* 2000). It is often agreed upon that no single test species is most sensitive for all systems, even among a group of taxonomically related organisms measured with the same endpoint (Nielson *et al.,* 1990). Many researchers agree that greater attention should be given to examining species that differ physiologically or in their ecological function (Hodson and Blunt, 1981; Sloof *et al.,* 1983; Nielson *et al.,* 1990).

Many arguments have been made for the importance of using plants in toxicity tests. Roy and Hanninen (1994) note the ability of high plants to act as *in situ* biomonitors due to their abundance and limited mobility. Ecologists note the critical role played by plants in nutrient cycling, increasing sedimentation, reduction of erosion, and soil and sediment stabilization as well as their ability to provide oxygen and organic substances, on which most life forms depend (Louvar and Louvar, 1998; Freemark and Boutin, 1994; Benenati, 1990; Biernacki *et al.,* 1997; Fairchild, 1998). Plants also provide food, shelter, shade and nesting habitats for other organisms, including insects, fish, invertebrates, mammals, amphibians, and birds (Freemark and Boutin, 1994; Biernacki *et al.,* 1997). Macrophytes also provide high quality detritus that is very important to benthic and pelagic food webs. This detritus may also buffer seasonal variations in available plankton (Wetzel, 1995). The critical role plants play in the food web highlights the importance of including them in standard toxicity testing.

Many reports indicate that lethal effects of toxicants on plants can have significant ecological and economic impacts (Altieri and Letourneau, 1982; Freemark and Boutin, 1994). Even sub-lethal effects can have significant consequences on food production and natural vegetation (Benenati, 1990; Hunsaker *et al.,* 1990; Weinstein *et al.,* 1990). Changes in aquatic plant dynamics can not only affect their own biota, but also the associated drinking water quality, as well as recreational and navigational water quality (Mohan and Hosetti, 1998). Despite the important functional roles and demonstrated ecological importance of plants in aquatic ecosystems, they tend to be overlooked in remediation studies and are rarely used in ecosystem studies, and rooted macrophytes are not required in standard screening procedures for introduction and registration of new chemicals (Biernacki *et al.,* 1997).

There has been extensive use of *Lemna* spp. for toxicity assessment. A summary of toxicity assessments to hazardous chemicals using *Lemna* spp. is included in Table 2.2. Data provided include plant used, contaminant or waste stream tested, effect concentration, biological indicator, toxicity endpoint, test duration and data source. Data listed in Table 2.2 represent a wide variety of organic and metal contaminants as well as several types of waste streams. Biological indicator typically used was frond count however, biomass production, oxygen production, chlorophyll content, PS II activity and enzyme activity were also used. These data demonstrate the differences in toxic effect concentration observed for different biological indicators, test duration, plant species and toxicity endpoint. For example, toxicity of 4-chlorophenol (4-CP) was assessed using

Table 2.2. Reported toxicity for *Lemna* species.

Species	Contaminant	Concentration	Biological	Toxicity	Citation
Lemna minor	Copper	0.8 mg/L	Frond count	EC50 7d	Bishop and Perry, 1981
Lemna minor	Sodium dodecyl sulfate	43 mg/L	Frond count	EC50 7d	Bishop and Perry, 1981
Lemna minor	Linear alkylbenzene sulfonate	2.7 mg/L	Frond count	EC50 7d	Bishop and Perry, 1981
Lemna minor	Alcohol ethoxylate	21 mg/L	Frond count	EC50 7d	Bishop and Perry, 1981
Lemna minor	Cetyl trimethyl ammonium chloride	0.1 mg/L	Frond count	EC50 7d	Bishop and Perry, 1981
Lemna minor	Diquat	0.0031 mg/L	Frond count	EC50 7d	Bishop and Perry, 1981
Lemna polyrrhiza	Cadmium	0.11 mg/L	growth	EC50 7d	Charpentier *et al.*, 1987
Lemna gibba	$K_2Cr_2O_7$	45 mg/L	Frond count	EC50 7d	Cowgill *et al.*, 1991
Lemna gibba	Diethanolamine	752 mg/L	Frond count	EC50 7d	Cowgill *et al.*, 1991
Lemna gibba	4-chlorophenol	55 mg/L	Frond count	EC50 7d	Cowgill *et al.*, 1991
Lemna	Phenol	226 mg/L	Frond count	EC50 7d	Cowgill *et al.*, 1991
Lemna gibba	Chlorobenzene	581 mg/L	Frond count	EC50 7d	Cowgill *et al.*, 1991
Lemna gibba	Chloroform	>1000 mg/L	Frond count	EC50 7d	Cowgill *et al.*, 1991
Lemna gibba	Acetone	10,158 mg/L	Frond count	EC50 7d	Cowgill *et al.*, 1991
Lemna gibba	Ethanol	4,432 mg/L	Frond count	EC50 7d	Cowgill *et al.*, 1991

Table 2.2. continued

Species	Contaminant	Concentration	Biological	Toxicity	Citation
Lemna minor	Diethanolamine	1,504 mg/L	frond count	EC50 7 d	Cowgill *et al.*, 1991
Lemna minor	4-chlorophenol	35 mg/L	frond count	EC50 7 d	Cowgill *et al.*, 1991
Lemna minor	Phenol	293 mg/L	frond count	EC50 7 d	Cowgill *et al.*, 1991
Lemna minor	Chlorobenzene	578 mg/L	frond count	EC50 7 d	Cowgill *et al.*, 1991
Lemna minor	Chloroform	>1,000 mg/L	frond count	EC50 7 d	Cowgill *et al.*, 1991
Lemna minor	$K_2Cr_2O_7$	30 mg/L	frond count	EC50 7 d	Cowgill *et al.*, 1991
Lemna minor	Acetone	11, 433 mg/L	frond count	EC50 7 d	Cowgill *et al.*, 1991
Lemna minor	Ethanol	4,875 mg/L	frond count	EC50 7 d	Cowgill *et al.*, 1991
Lemna minor	Phenol	540.5 μM	frond count	EC50 7 d	Ensley *et al.*, 1997
Lemna minor	4-chlorophenol	183.0 μM	frond count	EC50 7 d	Ensley *et al.*, 1997
Lemna minor	2,4-dichlorophenol	9.2 μM	frond count	EC50 7 d	Ensley *et al.*, 1997
Lemna minor	2,4,5-trichlorophenol	2.1 μM	frond count	EC50 7 d	Ensley *et al.*, 1997
Lemna minor	2,4,5,6-tetrachlorophenol	1.2 μM	frond count	EC50 7 d	Ensley *et al.*, 1997
Lemna minor	Pentachlorophenol	2.0 μM	frond count	EC50 7 d	Ensley *et al.*, 1997
Lemna minor	Alachlor	198 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Atrazine	153 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Bromoxynil	8,065 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Chlorsulfuron	0.7 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997

Table 2.2. continued

Species	Contaminant	Concentration	Biological	Toxicity	Citation
Lemna minor	Cyanazine	705 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Dicamba	>100,000 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Diquat	18 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	EPTC	7,512 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Metoachlor	343 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Metribuzin	37 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Metsulfuron	0.4 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Paraquat	51 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Simazine	166 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Triallate	>10,000 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Trifluralin	170 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	2,4-D	>100,000 ug/L	biomass	EC50 5 d	Fairchild *et al.*, 1997
Lemna minor	Anthracene	2 mg/L	biomass	EC50 5 d	Huang *et al.*, 1997
Lemna perpusilla	Spent caustic extraction liquor diluted 1:8	2.7%	% growth of control		Rowe *et al.*, 1982
Lemna minor	Undiluted effluent C1	6.1%	% growth of control		Sherry *et al.*, 1997
Lemna minor	Undiluted effluent C2	4.8%	% growth of control		Sherry *et al.*, 1997

Table 2.2. continued

Species	Contaminant	Concentration	Biological Indicator	Toxicity Endpoint	Citation
Lemna minor	Undiluted effluent C2	4.8%	% growth of control		Sherry *et al.*, 1997
Lemna minor	Undiluted effluent C3	1.4%	% growth of control		Sherry *et al.*, 1997
Lemna minor	Undiluted effluent C4	19.8%	% growth of control		Sherry *et al.*, 1997
Lemna minor	Undiluted effluent A1	58.1%	% growth of control		Sherry *et al.*, 1997
Lemna minor	Undiluted effluent A2	37.1%	% growth of control		Sherry *et al.*, 1997
Lemna minor	Undiluted effluent B1	56.7%	% growth of control		Sherry *et al.*, 1997
Lemna minor	Undiluted effluent B2	10.8%	% growth of control		Sherry *et al.*, 1997
Lemna minor	Effluent POTW-1 municipal	>100%	Frond number	Ch V*	Taradlsen and Nordberg-King, 1990
Lemna minor	Effluent POTW-2 municipal	>100%	Frond number	Ch V*	Taradlsen and Nordberg-King, 1990
Lemna minor	Effluent POTW-3 municipal	>100%	Frond number	Ch V*	Taradlsen and Nordberg-King, 1990

Table 2.2. continued

Species	Contaminant	Concentration	Biological Indicator	Toxicity Endpoint	Citation
Lemna minor	Effluent POTW-4 municipal	>100%	Frond number	Ch V*	Taradlsen and Nordberg-King, 1990
Lemna minor	Effluent POTW-5 municipal	>100%	Frond number	Ch V*	Taradlsen and Nordberg-King, 1990
Lemna minor	Effluent Ind-1 factory	35%	Frond number	Ch V*	Taradlsen and Nordberg-King, 1990
Lemna minor	Effluent Ind-2 mine	87%	Frond number	Ch V*	Taradlsen and Nordberg-King, 1990
Lemna minor	Effluent Ind-3 herbicide plant	<0.62%	Frond number	Ch V*	Taradlsen and Nordberg-King, 1990
Lemna minor	Effluent Ind-4 refinery	35%	Frond number	Ch V*	Taradlsen and [illegible]
Lemna minor	Atrazine	0.17 mg/L	Oxygen Production	EC50	Hughes *et al.*, 1988
Lemna minor	Pentachlorophenol	3.3 mg/L	Chlorophyll content	EC50	Huber *et al.*, 1982
Lemna minor	Pentachlorophenol	3.5 mg/L	Glutamate dehydrogenates activity	EC50	Huber *et al.*, 1982
Lemna minor	Pentachlorophenol	3.2 mg/L	Alanine aminotransferas	EC50	Huber *et al.*, 1982

Table 2.2. continued

Species	Contaminant	Concentration	Biological Indicator	Toxicity Endpoint	Citation
Lemna minor	Atrazine	56 ug/L	Frond count	EC50	Kirby and Sheahan, 1994
Lemna minor	Isoproturon	40 ug/L	Frond count	EC50	Kirby and Sheahan, 1994
Lemna minor	Mecoprop	5,147 ug/L	Frond count	EC50	Kirby and Sheahan, 1994
Lemna minor	2,4-dinitrophenol	7,100 ug/L	Frond count	EC50	Neilson *et al.*, 1990
Lemna minor	3,4-dinitrophenol	2,400 ug/L	Frond count	EC50	Neilson *et al.*, 1990
Lemna minor	2,4,5-trichlorophenol	730 ug/L	Frond count	EC50	Neilson *et al.*, 1990
Lemna minor	2,4,6-trichlorophenol	500 ug/L	Frond count	EC50	Neilson *et al.*, 1990
Lemna minor	2,4,,6-tribromophenol	760 ug/L	Frond count	EC50	Neilson *et al.*, 1990
Lemna perpusilla	Spent chlorination liquor diluted 1:8	69%	% growth of control	EC50	Rowe *et al.*, 1982
Lemna perpusilla	Spent chlorination liquor diluted 1:4	13%	% growth of control		Rowe *et al.*, 1982
Lemna perpusilla	Spent chlorination liquor diluted 1:4	0.54%	% growth of control		Rowe *et al.*, 1982
Lemna perpusilla	Spent chlorination liquor diluted 1:8	98%	% growth of control		Rowe *et al.*, 1982
Lemna perpusilla	Spent chlorination liquor diluted 1:8	17%	% growth of control		Rowe *et al.*, 1982

frond count over 7d with *Lemna gibba* and *Lemna minor* resulting in varied EC_{50} values of 55 and 35 mg/L, respectively (Cowgill et. al., 1991). Further, a toxicity test for 4-CP performed by Ensley and co-workers (frond count, 7d) provided a reduced EC_{50} value of 23 mg/L. Three tests which examined toxicity of atrazine to *Lemna minor* provided EC_{50} values of 153, 170, and 56 μg/L for a 5 d biomass test, a 5 d frond count test and a 10d frond count test, respectively (Fairchild *et al.,* 1997; Hughes *et al.,* 1988; Kirby and Sheahan; 1994). Disparity in EC_{50} values demonstrates the variation in results observed for changes in test duration, biological indicator, and test species.

There has been an initiative by several toxicologists to increase the use of floating vascular aquatic plants such as *Lemna* spp. due to simplicity of execution, technique reliability and repeatability, and ease of growing test species. Researchers also maintain that floating aquatics have an increased sensitivity to hydrophobic compounds that reside on the air-water interface. In addition to the extensive use of Lemna spp. in toxicity testing, there has been an initiative by toxicologists to begin use of submerged, rooted aquatic plants such as *Potamogeton pectinatus* and *Vallisneria americana* in standard testing of water and wastewater toxicity (Wang and Williams, 1988; Wang and Williams, 1990; Wang 1986; Taraldsen and Nordberg-King, 1990; Hall *et al.* , 1997; Biernacki *et al.,* 1997; Biernacki and Lovett-Doust, 1997; Fleming and Momot, 1988). Submerged aquatic plants have the advantage of being sensitive to water-column and sediment contamination. Routine use of plants in environmental assessment of toxic substances is warranted because components that may be practically nontoxic to fish, crustaceans, and

daphnids can injure and kill aquatic vegetation (Ashton and Crafts, 1981; Berry, 1984; Gersich and Mayes, 1986; Presing and Ponyi, 1986; Mayes *et al.,* 1987).

Plant Exposure Protocols

Throughout the literature, typical toxicity tests involve inoculation of a group of batch reactors containing a growth medium and a series of test contaminant concentrations with a known biomass of test species (Hughes *et al.,* 1988). Variations on this set-up include flow-through experiments, renewal experiments, field studies and experiments involving an initial exposure followed by a recovery period (Hedtke and Arthur, 1985; Hedtke *et al.,* 1986; Davis, 1981; Mohan and Hosseti, 1999; Brock *et al.,* 1992). Batch reactors are simple, economical systems that do not require maintenance. A batch set-up has many disadvantages, including exposure concentration changes with time due to chemical and biological processes, and higher nutrient levels are required to maintain adequate levels of growth. Batch tests are especially useful for screening unknown samples and samples with high levels of toxicants. Flow-through systems are more complex, but allow a constant level of contaminant exposure, thus they are more widely used with volatile or biodegradable contaminants. Populations tested in flow-through systems may be adversely affected, because of high flow-rates, intensity of contaminant, and crowding effects (Walbridge, 1977; Bishop and Perry, 1981; Mohan and Hosseti, 1999).

The literature suggests that the method of exposure must be taken into account for all reported values. Intensity of the effect of a contaminant is due not only to the concentration of the contaminant, but also the plant-to-contaminant ratio (Ensley *et al.,*

1997). Test duration is also important to test results, as it is a parameter that is directly tied to biological response indicator and toxicity assessment endpoint (Hughes *et al.,* 1988). Larson (1991) showed that test duration is critical to toxic responses, as several compounds have an initial inhibitory effect followed by a stimulatory effect, while others have an initial stimulatory effect followed by an inhibitory effect. Another important consideration noted in the literature is whether the test chamber is open or closed to the atmosphere. A closed chamber is important for volatile contaminants, and avoidance of microbial contaminants, whereas photosynthetic inhibition because of excess oxygen has been noted in closed systems (Hill *et al.,* 1984). Plant associated bacterial populations can also influence results, making careful monitoring for bacterial presence important (Larson, 1991). Contaminant pre-exposure is another variable that can significantly affect the stress level of organisms tested (Nielson *et al.,* 1990).

Toxic Effect Endpoints

There are many toxicity endpoints used to quantify toxic effects of contaminants. Commonly, results are expressed by determining if test concentrations cause significant differences in the health of the plant compared to a control, with observations of plant health taken after a given length of toxicant exposure. The highest test concentration where growth does not significantly differ from a control is termed no-observed effect concentration (NOEC), while the lowest concentration where a response significantly differs from a control is exhibited is termed lowest observed effects concentration (LOEC). LC_{50} is the concentration of a toxin in the exposure medium that is lethal to 50% of the organisms, while the LD_{50} is the dose of a toxin that is lethal to 50% of the

test organisms for the test duration. A test concentration causing 50% inhibition of growth relative to a control is termed 50% effect concentration, EC_{50} (Louvar and Louvar, 1998; Hughes *et al.,* 1988).

Some researchers have questioned the validity of such benchmarks *in situ*ations where normal growth rates can be reestablished once the toxic stress is removed (Bishop and Perry, 1981). Toxic effects to plants in laboratory testing can be temporary or permanent depending on the type of toxicant, concentration, route of exposure, plant species, life stage, health and other factors (Hughes *et al.,* 1988). These arguments lead to the development of a test procedure to determine phytocidal or phytostatic concentrations with procedures employing a five-day exposure followed by a nine-day recovery period. The phytostatic concentration is defined as the concentration that allows no net growth of the population of test organisms during exposure to the contaminant and allows net growth during the recovery period. The phytocidal concentration is the lowest concentration tested that allows no net increase in population density during either the exposure or recovery period (Hughes *et al.,* 1988; Payne and Hall, 1979).

pH Effects

Media pH plays a critical role in the toxic effects of ionizable contaminants to aquatic organisms (Saarikoski and Viluksela, 1981; Hedtke *et al.,* 1986). For weak acids and phenols, anionic forms are less toxic than their protonated counterparts (Svenson and Zhang, 1995; Saarikoski and Viluksela, 1981; Nielson *et al.,* 1990). It has been shown that test organisms often change the composition of the test medium, through the release

of organics and the uptake of nutrients (Peterson, 1991). Medium changes often result in a pH change, and therefore modifies the speciation of ionic toxicants, possibly altering the severity of the toxic effect. However, many researchers do not report any pH measurements associated with their toxicity tests, and often use media with poor buffer capacity. The influence of pH on toxicity in a system can go unnoticed, with no concern for a potential pH shift (Nielson *et al.,* 1990). Unreported pH values make toxic effect comparisons throughout the literature impossible.

The drastic effect that pH has on toxicity is clearly demonstrated in studies where researchers utilize a strong buffer system to control media pH for exploration of toxic effects of protolyzing compounds. Nielson *et al.* (1990) used several non-toxic buffers to control pH throughout the neutral range, and examine pH effect on toxicity using four test species: an aquatic vertebrate, *Brachydanio rerio;* an aquatic invertebrate*, Daphnia magna*; an aquatic plant, *Lemna minor;* and MICROTOX bacteria. All four species showed drastically different toxic effects for a given contaminant concentration depending on the pH of the media. The EC_{50} for *Lemna minor* exposed to 2,4,5-trichlorophenol shifted from 560 μg/L at pH 5.8 to 2,200 μg/L at pH 7.5. Likewise, 2,4,5-trichlorophenol toxicity to *D. manga* undergoes almost an order of magnitude change when the pH increases from 6.2 to 8.2, shifting the LC_{50} from 290 to 2,600 μg/L (Nielson *et al.,* 1990).

Most studies that note a pH influence have not proceeded further than the observation that an increased pH decreases toxicity of acidic compounds and increases toxicity of

basic compounds. Few attempts have been made to explain the cause of pH dependant toxicity. This analysis is critical for result comparison throughout the literature and the application of toxicity data to accumulation and toxicity of ionizable pollutants in various recipient waters, as many natural waters vary substantially in pH (Nielson *et al.,* 1990; Saarikoski and Viluksela, 1981). Several authors have proposed toxicity dependence on the pK_a of a test compound and the pH at which it is examined (Dalela *et al.,* 1980; Holcombe *et al.,* 1980; Kaila and Saariskoski, 1977). In one study with *Poecilia reticulata*, it was concluded that neither the molecular form exclusively, nor the molecular and ionic forms, could explain the toxicity of some chlorophenols (Saarikoski and Viluksela, 1981). In a similar study performed using MICROTOX, Svenson and Zhang (1995) concluded that a simple model of a toxic effect of a phenol and a much less toxic phenolate can be supported. However, the authors note that five of the six chlorophenols studied gave no indication of a contribution from the toxicity of the phenolate. Restrictions on test conditions are cited as potential reasons for limited results showing phenate toxicity. Another study explored a model where toxic effects were due only to free phenol, finding that toxic concentrations did not depend linearly on the concentration of free phenol for four species studied (Nielson *et al.,* 1990). Conclusions drawn from this extensive work indicate that toxicity depends not only on pK_a and media pH, but also by efficiency of transport into the cell. These results supported indications by numerous investigators of an influence on toxic effects due to transport efficiency as well as the pK_a of the contaminant and pH of the media (Blackman *et al.,* 1955a; Blackman *et al.,* 1955b; Saarikoski and Viuksela, 1982; Part, 1989).

Attempts have been made to develop a molecular scale model for uncoupling toxic effects of phenolic compounds. Escher *et al.* (1999) developed a model to predict toxic effects of phenols tied to membrane interactions, using *Rhodobacter sphaeroides* as a model species. The model was designed to predict the intrinsic uncoupling activity of a phenolic compound given a set of conditions. It separated uncoupling activity into contributions of the membrane concentration, speciation, and intrinsic activity. Input parameters included experimental data for relaxation of the membrane potential, biomembrane-water distribution constants of phenol and phenoxide species, acidity constant of phenol, translocation rate constant, and heterodimer formation constant of the phenolic species. The experimental data were described by an extended "shuttle mechanism" model where the rate of diffusion of the phenoxide and or a phenoxide/phenol-heterodimer species through the membrane determined the rate of diffusion of the electrochemical gradient (Escher *et al.,* 1996). While the research discussed herein concentrates on macro-scale plant systems, this model of molecular scale inhibition of a microbial system is important to understanding large-scale toxic effects.

Biological Indicators

Biological indicators are defined as measurements of fluids, cells or tissues that indicate the presence of contaminants or the magnitude of the host response (McCarthy and Shugart, 1990). Important criteria for usefulness of a biological indicator include sensitivity, reliability, feasibility, and applicability to the hazardous environment being tested (Louvar and Louvar, 1998). Numerous indicators are used to assess the health of a

plant depending on morphology of test species, sampling requirements and whether destructive or non-destructive measurements are required. Many types of biological indicators are utilized in the literature to assess contaminant effects.

Pollutants affect biological systems at many levels, initially changing structure and/or functional properties of molecules essential to cellular activities. These molecular changes then affect the structure and function of organelles and cells, which alters the physiological state of the organism. Physiological status changes influence energy allocation, which can affect growth and reproduction. Altered patterns of growth and reproduction, affecting a number of individuals, produces effects in populations. Changes in population can influence community structure. Based on this hierarchy of effects, biological indicators are generally grouped into three broad levels: biochemical-molecular level, cell and tissue level, and organism level. An appropriate choice in biological indicator is important because extrapolations of observations made at one level to predict effects at levels higher or lower in the hierarchy are generally not possible (Shugart, 1996).

Organism Level Indicators. Organism level indicators are common in toxicity assessment because of simplicity of measurement, as well as minimal time and equipment requirements. For aquatic plant toxicity, organism level indicators involve a physical measurement comparison of a plant or a part of a plant. Plant number, frond count, leaf number, number of flowers, number of raments, biomass per rament, leaf to root biomass ratio and leaf to root surface area ratio are common ways to account for the

growth of larger groups of plants (Biernacki *et al.,* 1995; Bishop and Perry, 1981; Charpentier *et al.,* 1987; Fairchild *et al.,* 1997; King and Coley, 1985; Kirby and Sheahan, 1994; Teisseire *et al.,* 1997; Lockhart *et al.,* 1981; Watkins and Hammerschlag, 1984; Elakovich, 1999; Bowman *et al.,* 1997; Sharmra *et al.,* 1997; Ensley *et al.,* 1997). Other commonly used physical measurements are used to determine inhibition at the organismal level such as fresh weight, dry weight, root dry weight, shoot length, root length, plant length, leaf area index, leaf length, and number of rhizome tips (Huang, 1991; Fleming *et al.,* 1991; Lockhart *et al.,* 1981; Zhihui and Tianyi, 1998; Kirby and Sheahan, 1994; Bishop and Perry, 1981; Hall *et al.,* 1997; Ramachandrian *et al.,* 1984; Hughes *et al.,* 1988; Fleming and Momot, 1988; Watkins and Hammerschlag, 1984; Bowman *et al.,* 1997).

Many physical biological indicators are not relevant for use with all species of plants, as plants are not all physiologically similar. What may be an appropriate indicator of health for one type of plant may be irrelevant for another type. For example, measurements such as number of raments and number of rhizome tips are options for rament forming plants with full root systems such *as Vallisneria americana*, but are not relevant to floating aquatics such as *Azolla* spp. and *Lemna* spp. as the roots systems of many floating species often involve only one root. Number of leaves per plant, number of flowers per plant and seed germination are often not appropriate choices as they are heavily dependent on lifecycle stage and seasonal variations.

Root length or root biomass measurements are an option for floating aquatic plants where effects on roots are considered to be the most sensitive measurement of phytotoxicity (Bishop and Perry, 1981). However, roots are often a negligible portion of the total biomass and are sometimes too minute to measure. Root measurement becomes extremely tedious for large groups of plants, and because the root is often an extremely fragile portion of a plant, accurate root length measurements are difficult and time consuming. Some aquatic plants such as *Lemna* spp. can adsorb nutrients through the lower portion of a frond and can function efficiently in a medium that completely inhibits root elongation. Toxicity studies have been performed on *Lemna* spp. with the root removed, discrediting root measurements as biological indicator options (Bishop and Perry, 1981; Tardalson and Nordberg-King, 1990). Shoot length is also used as a biological indicator in plants that contain stems, but this is considered to be the least sensitive physical parameter (Bishop and Perry, 1981). Both root and shoot length measurements are destructive sampling techniques and require sacrificial sampling of planted mesocosms, which is often an experimental inconvenience.

For small quantities of *Lemna* spp., many authors cite frond count as the most reliable and sensitive indicator of toxicity (Elakovich, 1999; Bishop and Perry, 1981; Fairchild *et al.,* 1997; King and Coley, 1985; Kirby and Sheahan, 1994; Teisseire *et al.,* 1997; Lockhart *et al.,* 1981; Watkins and Hammerschlag, 1984; Elakovich, 1999). It is a simple, rapid, and non-destructive biological indicator. Typical toxicity tests utilizing frond counts as a biological indicator begin with 10 to 30 fronds and use short exposure periods such as 96-hour tests. Disadvantages of frond count as a biological indicator

include the fact that frond count is irrelevant to biomass amount. Under stress, *Lemna* spp. will produce small buds that only comprise around 5% of the total mass. These buds are counted as a full sized frond, grossly underestimating toxic effects (Wang, 1990). Additionally, frond counts do not distinguish between dead and live fronds, often producing misleading results (Wang and Williams, 1988; Mohan and Hosseti, 1998). While many authors find frond counts to be an appropriate biological indicator for use with very small amounts of *Lemna* spp., this method becomes impossible if initial biomass increases only one order of magnitude or exposure time extends past 96 hours. Additionally, frond count is not a biological indicator readily adaptable to other plant species.

Raw biomass measurements such as fresh weight and dry weight can be used with virtually any species of plant. These two biological indicators test whole plant effects and are considered to have intermediate sensitivity as a biological indicator. One disadvantage associated with both methods is that there is no distinction between live and dead tissue (Wang and Williams, 1988). Fresh weight is a simple, non-destructive method for roughly determining amount of biomass. However, this indicator is subject to high variability as an inconsistent amount of water is carried with the plants into each measurement. Blotting the plants on a paper towel before weighing is a technique that minimizes this variability, but there is still a great deal of fluctuation in water removed through blotting, returning the problem of inconstant amounts of water carry-over. Wet weight and dry weight techniques are problematic when used with large populations, as the changes in biomass are often not detectable compared to background mass. Dry

weight is a similar measurement used throughout the literature where the plant material is dried to remove all water prior to weighing. Dry weight measurements are inherently tied to wet weight measurements, as analysis performed compares initial weight to final weight, and initial weight must be measured as wet weight. Dry weight measurements can also be misleading as they often reflect changes in starch content rather than growth (Bishop and Perry, 1981).

Cellular Level Indicators. Cell and tissue level responses are another common way to measure plant inhibition. These measurements are directly related to primary plant processes, often giving a more immediate and sensitive response to toxic effects. Chlorophyll content, cartenoid content, soluble carbohydrate content, adenosine triphosphate content, protein content, nitrate and phosphate uptake, ethylene emissions, Kjeldahl Nitrogen measurements and ion accumulation are methods used to approximate amount and activity of biomass present (Gupta and Chandra, 1994; Robinson and Wellburn, 1991; Kirby and Sheahan, 1994; Taraldson and Nordberg-King, 1990; Teisseire *et al.,* 1997; Zhihui and Tianyi, 1998; Miles, 1990; Krupa *et al.,* 1993; Yerkes *et al.,* 1990; Peterson, 1991; Breteler *et al.,* 1991; Nwosu *et al.,* 1991; Walsh *et al.,* 1991; Velagateli *et al.,* 1990; Wilson and Al Handani, 1997; Wolfenden *et al.,* 1988; Zhihui and Tianyi, 1998; Cowgill and Milazzo, 1989; Caux *et al.,* 1988). This group of assays gives a number designating live tissue per biomass (*e.g.,* cartenoids per gram plant), designed to relate plant tissue health.

Carbon assimilation and oxygen production are two common assays used to measure net photosynthetic activity of plant systems, with photosynthetic output reportedly paralleling biomass production (Fleming *et al.,* 1995; Kemp *et al.,* 1986; Huang, 1997; Lockhart *et al.,* 1981; Ramachandrian *et al.,* 1984; Vollenweider, 1969; Hill *et al.* 1984). High levels of oxygen have been shown to inhibit plant processes (Hill *et al.* 1984; Vollenweider, 1969). Other concerns with using oxygen production as a biological indicator include the possibility of oxygen bubbles trapped in the media, as well as pressure or temperature fluctuations (Vollenweider, 1969). There is evidence that certain types of algae do not maintain the same respiration rates in light and dark systems, making gross photosynthesis calculations invalid. Cyclic photophosphorylation allows plants to produce and utilize oxygen without exchanging the oxygen with the atmosphere, further increasing error in oxygenic measurements (Vollenwelder, 1969; Kemp *et al.,* 1986). Other problems cited are oxygen storage in the lacunal system, and release from roots to the external rhizosphere (Kemp *et al.,* 1986).

Error sources associated with carbon fixation as a measure of photosynthetic activity include the fact that all reactions involved in incorporation of bicarbonate are not connected with photosynthesis, thus carbon fixation is an overestimate of photosynthetic activity. Additionally, incorporation of organic matter, or 'dark' carboxylation reactions is often suppressed by light, and therefore cannot be corrected for by subtracting carbon fixation in dark systems. Interactions between photosynthesis and respiration are complex, where products of photosynthesis may be used preferentially in respiration. In

experiments lasting more than 24 hours, the loss of photosynthetically fixed carbon by respiration may be appreciable.

Most tests involving both oxygen evolution and carbon fixation measurements demonstrate an excellent correlation, except under high light intensity and in unconcentrated natural populations (Vollenweider, 1969; Kemp *et al.,* 1986). Kemp (1986) suggested that the above-cited sources of error for both carbon fixation and oxygen evolution represented minimal sources of error for measuring productivity of aquatic plants.

Chlorophyll fluorescence can be used to sense changes in the electron transport system. Researchers consider chlorophyll fluorescence to be a highly sensitive and telling bioindicator, as changes in lipid soluble compounds or in highly reducing or oxidizing compounds will affect characteristics of electron transport and therefore will be immediately identified by changes in chlorophyll fluorescence. Additionally, changes in carbon assimilation will change the NADPH pool, eventually changing chlorophyll fluorescence activity (Ruth, 1996; Krupa *et al.,* 1993; Miles, 1990; Velagateli *et al.,* 1990; Caux *et al.,* 1988). Chlorophyll fluorescence assay is non-destructive, but requires highly specialized equipment (Velagateli *et al.,* 1990). Several other specific assessments of photosystem activity and electron transport can also be used to determine the mode of inhibition of a contaminant (Huang, 1997; Judy *et al.,* 1991; Judy *et al.,* 1990; Kramer and Crofts, 1990; Kramer *et al.,* 1990; Kramer and Crofts, 1996; Schnabel and Youngman, 1987).

Growth retarding enzymes such as glycolate oxidase are used as growth rate indicators, where the less enzyme is present the more quickly plant mass is produced (Einor *et al.,* 1989). Huber (1982) used chlorophyll *a* content, glutamate dehydrogenase levels and alanine aminotransferase activity to evaluate herbicides indicated to potentially block protein building abilities in plants. Other specific assays determine plant content of stressor proteins such as peroxidases, catalyase, anthocyanin or mellothionein to give an indication of plant stress levels (Shugart, 1996; Leather and Einhellig 1985, Byl and Klaine, 1991; Teisseire *et al.,* 1997; Mohan and Hosseti, 1999). For example, Roy and Hanninen (1994) assessed stressor proteins peroxidase, ascorbate peroxidase, glutathione reductase, glutathione s-transferase and superoxide dismutase to measure stress levels in the aquatic plant, *Eichhornia crassipes*.

Several commonly used indicators such as chlorophyll fluorescence assays and stressor enzyme content assays give an indication of plant stress caused by environmental effects. This type of test is used to indicate subtle effects on cell and tissue level processes, but does not give an indication of serious offenses to cellular integrity and cannot be accurately used as plants approach death. Additionally, these indicators are prone to high variability as they are inherently based on fresh weight measurements.

In a comparison of many types of biological indicators, Cowgill and Milazzo (1989) indicated that physical measurements are all highly related to each other, but are difficult to relate to Kjeldahl Nitrogen measurements or chlorophyll content. According to Mohan and Hosseti (1998) changes in carbohydrate, protein, proline, and chlorophyll content as

well as enzyme activity (protease, catalase, and peroxidase) is a better tool for toxic responses of duckweed to heavy metals than is a population estimate such as frond number or plant number. It has also been indicated that dry weight biological indicator is the least time consuming, and least subject to human error (Cowgill and Milazzo, 1989). Mohan and Hosseti (1998) indicated that a strong correlation between respiration and peroxidase and catalase activity has been developed for heavy metal toxicity. In a review of toxicity methods, Mohan and Hosseti (1999) state that the most sensitive and accurate biological indicators include viable biomass and physiological activity: adenosine triphosphate, fluorescence emission, ^{14}C uptake, enzyme activity and oxygen activity. While these are all important to consider when selecting a biological indicator it appears that few options have been ruled out by this assessment.

Characterization of Chlorinated Phenols

All of the nineteen possible chlorinated phenols are commercially available, many of which are highly toxic and persistent in the environment, and as a result are prominent on the EPA's List of Priority Pollutants. Of the nineteen possible chlorophenols, pentachlorophenol (PCP), 2,4,5-trichlorophenol (2,4,5-TCP), 2,4-dichlorophenol (2,4-DCP), 2-chlorophenol (2-CP) and 4-chlorophenol (4-CP) are considered to be the most important because of high levels of production and use. Historical applications of chlorinated phenols include use as wood preservatives, pesticides, bactericides and fungicides, as a precursor for the production of herbicides as well as many other uses (Mark *et al.,* 1978; Ensley *et al.,* 1997). Chlorophenols are well suited to biocidal applications since they are somewhat toxic and resistant to microbial degradation. The

compounds 2,4,5-trichlorophenol and 2,4-dichlorophenol are described in further detail to provide an example of uses, as well as physical and chemical properties.

Industrial Use of Chlorophenols

2,4,5-TCP is used most commonly as an intermediate in the manufacture of herbicides 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2-(2,4,5-trichloropehnoxy)ethyl 2,2-dichloropropionate (ebron) and 2-(2,4,5-trichlorophenosy)propionic acid (silvex), the germicide hexachlorophene and the insecticides trichloronate and Fenchlorphos. Marketed by the Dow Chemical Company, 2,4,5-TCP is also used as an antifungal agent in applications such as in adhesives, as a preservative in polyvinyl acetate emulsions, in the automotive industry to preserve rubber gaskets, and in the textiles industry to preserve emulsions used in the rayon industry. The sodium salt of 2,4,5-TCP is also used in adhesive applications, in cooling water as an inhibitor of microbial growth, in foundry core wash to prevent breakdown of oils and scum formation, in leather dressing and finishes to prevent decomposition of nitrogenous compounds and in metal working fluids to prevent breakdown of oils, emulsifying agents, and other components (Mark et. al.., 1978). The main commercial interest in 2,4-dichlorophenol is use as a precursor for the production of 2,4-dichlorophenoxyacetic acid (2,4-D), a widely used herbicide that remains in production in the United States today (Mark et. al., 1978). The formulation of Agent Orange, a defoliant that was heavily used in tropical settings in the 1960's and 1970's, particularly in the Vietnam conflict, involves a 50/50 mixture of 2,4,5-T and 2,4-D. (Fallon *et al.,* 1994*).*

Relevant Chemical and Physical Properties of Chlorophenols

Vapor Pressure. Vapor pressure of a compound is the pressure of the vapor of a compound at equilibrium with its pure condensed phase (Schwarzenbach *et al.,* 1993). This parameter gives an indication of the tendency of a chemical to transfer to and from the gas phase by predicting the equilibrium distribution of the compound. Vapor pressure of TCP was estimated at 25° C using the Clayperon Equation, where the Kistakowsky-Fistine Correction was used to correct for polar effects. As TCP is a solid at 25° C, boiling (T_b) and melting temperatures (T_m) were necessary in calculations, where values used were T_b = 526.1 K and T_m = 341.1 K (Howard and Meylan, 1997). In TCP the –OH group dominates substituent effects causing the compound to be polar with a H-bonding effect, thus a correction factor of K_F = 1.3, found for an alcohol, was used. The Vapor Pressure was estimated to be 1.043E-6 atm at 25° C.

A relationship given by National Institute of Standards and Technology, taken from data reported by Stull (1947) indicated that the vapor pressure of 2,4,5-TCP at 25° C is 5.23E-5 atm. Several reports of the vapor pressure of 2,4,5-TCP were given by Mackay (1985) in the range of 2.89E-5 atm to 7.54E-5 atm. Howard and Meylan (1997) report the vapor pressure of 2,4,5-TCP as 2.63E-5 atm. A summary of values found in the literature compared with estimated values is found in Table 2.3. Data shown indicate that the estimated vapor pressure compares well with data reported in the literature, where values vary within one order of magnitude.

Table 2.3. Comparison of estimated vapor pressure of 2,4,5-TCP with literature data.

Vapor Pressure	Source
1.043E-6 atm at 25°C	Estimation based on Clayperon Equation, T_b and Kistakowski-Fishtine Correction
2.632E-5 atm at 25°C	Bidleman and Renberg, 1985 in Howard and Meylan, 1997
5.263E-1 atm at 225°C	Verschueren, 1996
6.602E-5 atm at 25°C	Stull, 1947, in Mackay *et al.,* 1995
3.01E-5 atm at 25°C	Chiou and Freed 1977, in Mackay *et al.,* 1995
2.44E-5 atm at 25°C	Cheung, 1984 in Mackay *et al.,* 1995
6.51E-5 atm at 25°C	Liquid value, Antione eqn., Weast 1972-73, in Mackay *et al.,* 1995
6.04E-5 atm at 25°C	Subcooled liquid, Hamilton 1980, in Mackay *et al.,* 1995
2.89E-5 atm at 25°C	Leuenberger *et al.,* 1985, in Mackay *et al.,* 1995
7.54E-5 atm at 25°C	Extrapolated-Antione eqn., Stephenson and Malanowski, 1987, in Mackay *et al.,* 1995
2.89E-5 atm at 25°C	Howard, 1991, in Mackay *et al.,* 1995

The vapor pressure of 2,4-DCP was reported by Howard and Meylan (1997) to be 8.82E-3 atm at 25ºC and many values were reported by Mackay and co-workers (1985) ranging from 7.8E-5 to 1.58E-4 atm at 25ºC. The vapor pressure of 2,4-DCP was estimated to be 3.12E-5 atm using the Clayperon Equation and the Kistakowsky-Fistine Correction with 42ºC used for the melting temperature and 209ºC used for the boiling temperature (Howard and Meylan, 1997; Mackay *et al.,* 1987). Thus, the vapor pressure of 2,4-DCP is comparable with that of 2,4,5-TCP, both of which are relatively low values.

Acidity. Ionizable compounds have very different properties and reactivities in their neutral and charged states. The acidity constant (pK_a) is the pH where the protonated and deprotonated form of the compound are equal in solution, therefore a pKa in the range of environmentally relevant pH values indicates that both the neutral and charged species will be available in aqueous solution. 2,4,5-TCP has a near neutral acidity constant, with a reported pKa values ranging from 6.72 to 7.43 (Table 2.4). The most commonly reported pKa reported is 7.0 (Blackman *et al.,* 1955a; Blackman *et al.,* 1955b; Howard and Meylan, 1997; Mackay *et al.,* 1985). The Hammett Correlation was used to estimate the pKa of 2,4,5-TCP, where a values of 7.29 and 7.45 were found using different combinations of substituent additions. Additionally, the pKa was determined experimentally to be ~7 for a high ionic strength solution using the different response of an HPLC to the ionized and neutral forms of 2,4,5-TCP (Figure 2.3). These data indicate that both species of 2,4,5-TCP will be present in almost all natural waters. The pKa for 2,4-DCP was reported to be 7.89 by Howard and Meylan (1997) and values ranging from 7.68 to 8.09 were reported by Mackay and coworkers (1987) with a median value of 7.9.

Table 2.4. Comparison of estimated acidity constant of 2,4,5-TCP with literature data.

Acidity Constant (pK_a)	Source
7.29	Calculated using Hammet correlation
6.94	Schellenberg *et al.,* 1984 in Howard and Meylan, 1997
7.44	Kirk-Othmer, 1974
7.00	Blackman *et al.,* 1955; Sillen and Martell, 1971; Kaiser *et al.,* 1984 in Mackay *et al.,* 1985
7.07	Farquharson *et al.,* 1958; Saarikoski and Viluksela, 1982; Renner, 1990 in Mackay *et al.,* 1985
7.43	Doedens, 1967; Jones, 1981; Bintien and Devillers, 1994 in Mackay *et al.,* 1985
6.72	Ugland *et al.,* 1981; Dean 1985; Lagas, 1988; Renner, 1990; Ma *et al.,* 1993 in Mackay e t al, 1985
6.90	Hoigne and Bader, 1983 in Mackay *et al.,* 1985
6.83	Nendzan and Seydel, 1988 in Mackay *et al.,* 1985

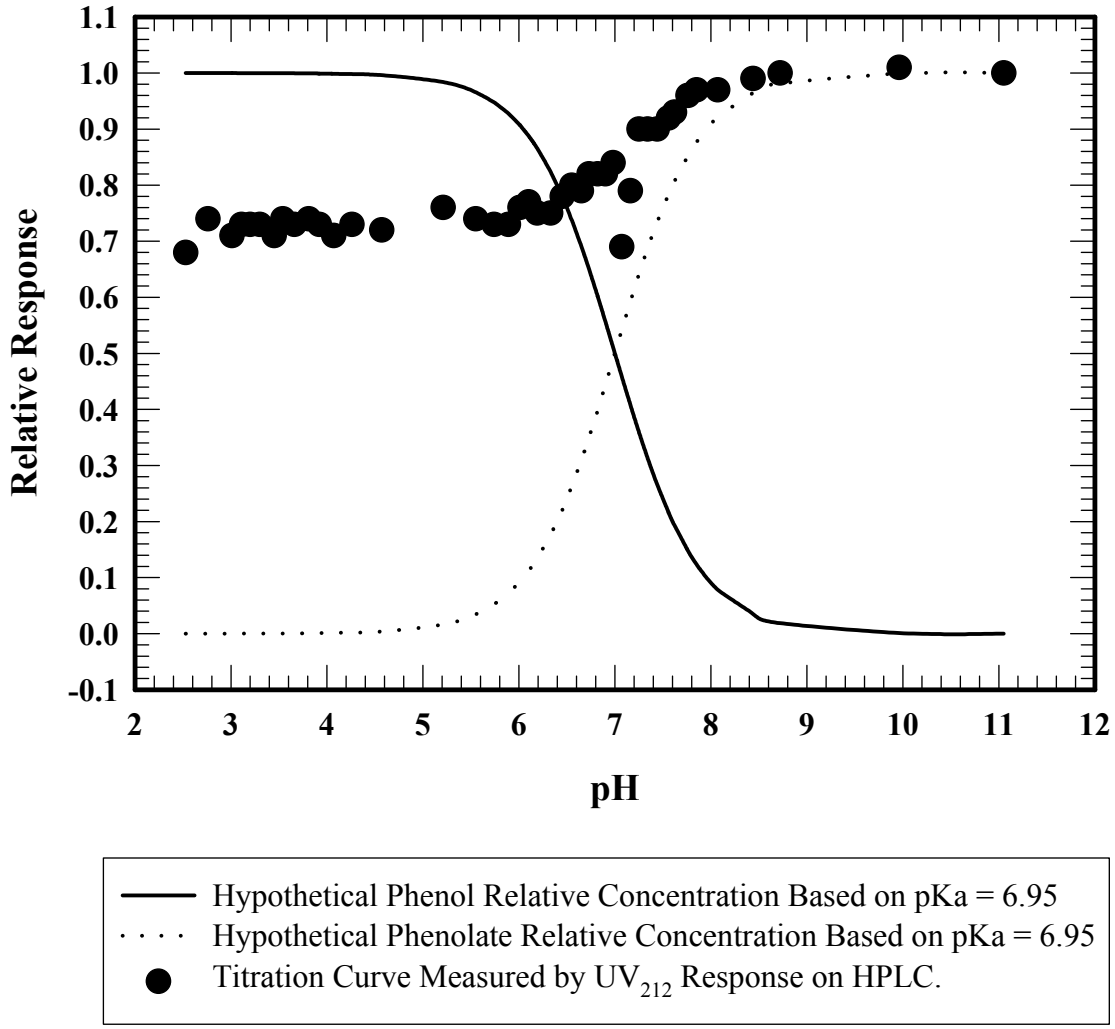


Figure 2.3. UV_{212} response as a function of pH in media modified for pH control.

Thus both the protonated and unprotonated forms of 2,4-DCP are relevant in environmental systems. The pKa is a very important parameter for ionizable compounds such as 2,4,5-TCP and 2,4-DCP because is predicts the amount of the compound in the protonated and deprotonated form. The speciation of these compounds is critical to solubility, volatilization, and partitioning. The deprotonated forms are much more hydrophilic and therefore dissolve in water more easily, and do not partition readily into air or organic matter. As a result, the toxicity of 2,4,5-TCP to aquatic organisms has been shown to be dependent on the pH of the system under which the organisms are exposed (Svenson and Zhang, 1995; Saarikoski and Viluksela, 1981; Neilson *et al.,* 1990). Consideration of the pH is essential to the behavior and fate of chlorophenols in the environment.

Solubility. Aqueous solubility is defined as the abundance of chemical per unit volume in the aqueous phase when the solution is in equilibrium with the pure phase (Schwarzenbach *et al.,* 1993). This parameter is a critical factor in determining the environmental fate of the compound. The solubility of 2,4,5-TCP in water has reported as 1 - 1.2 g/L throughout the literature, with values summarized in Table 2.5 (Mark *et al.,* 1978; Mackay *et al.,* 1985). The solubility of 2,4-DCP in water at 20ºC was reported to

Table 2.5. Comparison of literature values of the aqueous solubility of 2,4,5-TCP.

Aqueous Solubility (g/L)	Source
0.948	Shake-flask UV at pH 5.1, Blackman *et al.,* 1955 in Mackay *et al.,* 1995
1.19	Verschueren, 1996
0.982	Selected, Cheung 1984, in Mackay *et al.,* 1995
0.990	Quoted, LeBlanc 1984, in Mackay *et al.,* 1995
1.20	Recommended at pH 5.1, IUPAC, in Mackay *et al.,* 1995
0.70	8°C, Leuenberger *et al.,* 1985, in Mackay *et al.,* 1995
0.96	Selected, Suntio *et al.,* 1988, in Mackay *et al.,* 1995
1.202	Quoted, Isnard and Lambert 1988, in Mackay *et al.,* 1995
0.982	Quoted, Howard 1991, in Mackay *et al.,* 1995
1.200	Quoted, Muller and Klein 1992, in Mackay *et al.,* 1995
0.649	Shake flask-HPLC/UV at pH 4.9, Ma *et al.,* 1993, in Mackay *et al.,* 1995

be 4.50 g/L and 4.47 – 6.19 g/L at 25ºC (Howard and Meylan, 1997; Mackay *et al.,* 1987).

Aqueous solubility of chlorinated phenols is dictated by several factors, including salt concentration and the pH of the aqueous medium. Calculations estimating the solubility of 2,4,5-TCP in an estuarine system indicate that increasing the salt concentration from pure water to 20 parts per thousand would lower the solubility of 2,4,5-TCP from 1.2g/L to 1.11 g/L. As 2,4,5-TCP and 2,4-DCP are ionizable in the range of environmentally relevant pH values, it should be noted that their aqueous solubilities are dependant on pH as the deprotonated form of the compound is much more hydrophilic than the protonated form.. The effect of pH on the aqueous solubility of 2,4,5-TCP is shown in Figure 2.4, where the aqueous solubility shifts two orders of magnitude from pH 6 to pH 9. This data set shows that pH plays a significant role in determining the aqueous solubility of 2,4,5-TCP, a factor that has been disregarded in many of the literature reports of aqueous solubility.

Volatilization. The Henry's Law constant, K_H, is a measure of the equilibrium phase distribution between the gas and aqueous phases when a compound is found in dilute aqueous solution (Schwarzenbach *et al.,* 1993). A simple calculation for determination of K_H is the ratio between vapor pressure and aqueous solubility. Using values commonly reported in the literature, K_H of 2,4,5-TCP equals 4.33E-3 atm·L·mol^{-1} and K_H of 2,4-DCP equals 1.13E-3 atm·L·mol^{-1} Several values of K_H for 2,4,5-TCP are given in the literature, ranging from 1.30E-3 atm·L·mol^{-1} to 5.82E-3 atm·L·mol^{-1} (Table 2.6). A

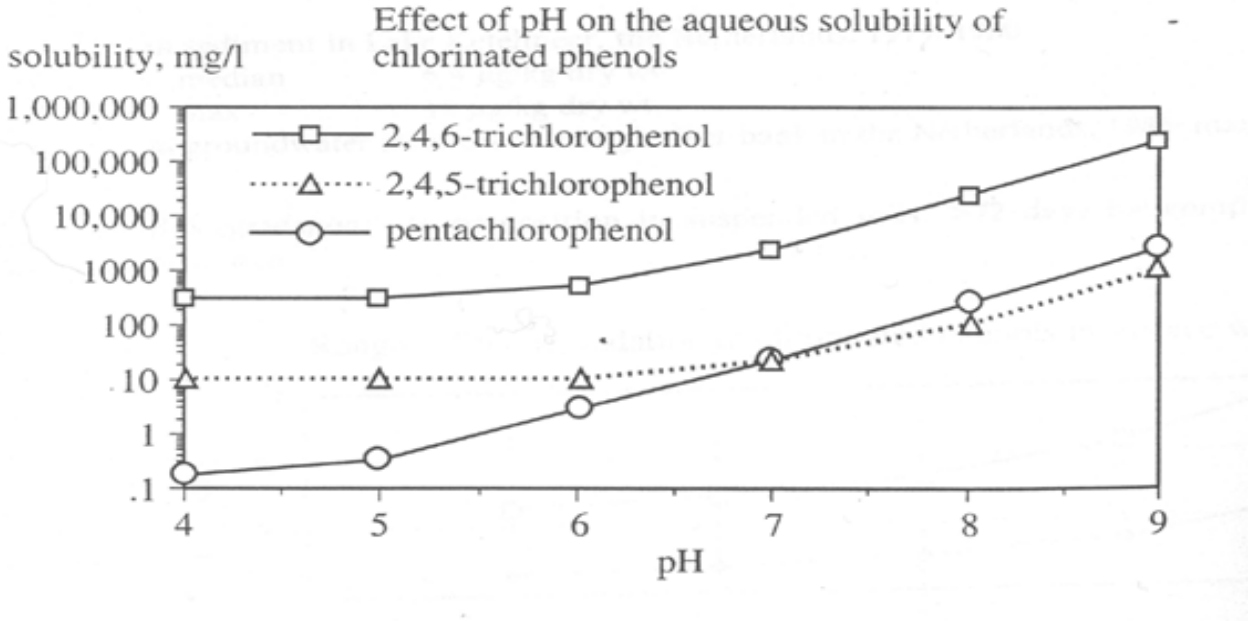


Figure 2.4. The effect of pH on the solubility of selected chlorinated phenols (from Verschueren, 1996).

Table 2.6. Comparison of estimated and literature values for Henry's Law constant of 2,4,5-TCP.

Henry's Law Constant ($atm \cdot L \cdot mol^{-1}$)	Source
2.281E-4	Estimated using Bond Contribution Method
4.33E-3	Estimated using literature values for Vapor Pressure and Aqueous Solubility
4.33E-3	Howard and Meylan, 1997
1.30E-3	8°C, Leuenberger *et al.,* 1985, in Mackay *et al.,* 1985
5.142E-3	Shiu *et al.,* 1994 in Mackay *et al.,* 1985
5.822E-3	Howard *et al.,* 1991, in Mackay *et al.,* 1985

range of K_H values for 2,4,-DCP (1.08E-3 – 4.29E-3 atm·L·mol^{-1}) were given in the literature (Howard and Meylan, 1997; Mackay *et al.,* 1987).

The Henry's Law constant is also affected by salt concentration and pH as it is a function of aqueous solubility. It should be noted that with high salt concentration, as in estuarine water, the aqueous solubility is decreased, therefore the K_H is increased and increasing partitioning into the atmosphere is observed. K_H is also an inverse function of pH, where the value of K_H will be high at low pH values due to a decreased aqueous solubility and the K_H will be low at high pH values, because of increased aqueous solubility. Just as aqueous solubility of 2,4,5-TCP increases two orders of magnitude in the pH range of 6 to 9 (Figure 2.4), K_H of 2,4,5-TCP decreases two orders of magnitude in this pH range.

Hydrophobicity. The octanol-water partitioning coefficient, K_{ow} is a ratio between the concentration of a compound in water to the concentration of the compound in octanol when these two immiscible liquids are in equilibrium with each other and the compound of interest (Schwarzenbach *et al.,* 1993). This parameter is a surrogate measure of the distribution of non-polar compounds between water and natural solids such as soils, sediments, and suspended particles or organisms and other naturally occurring organic material. The uptake of a compound into the octanol is considered to be proportional to the uptake the compound by an organism, or other naturally occurring organic phases. High K_{ow} values indicate hydrophobicity or preference of the organic phase, while low K_{ow} values indicate hydrophilicity or preference for the water phase.

The log K_{ow} of 2,4,5-TCP has been estimated as being 2.775 using linear a free energy relationship (LFER) developed for the aqueous solubility of substituted benzenes with polar substituents ($R^2 = 0.86$). The K_{ow} of 2,4,5-TCP was also estimated using the Structure-Fragment Contribution approach developed by Lyman (1982). These calculations resulted in log K_{ow} equal to 4.94, a value two orders of magnitude higher than that determined using LFER. Many K_{ow} values for 2,4,5-TCP have been reported in the literature ranging from 3.06 to 4.19, with the value of 3.72 found most commonly (Table 2.7). Estimates of K_{ow} for 2,4,5-TCP using the LFER and Structure-Fragment Contribution method are both outside the range of values observed in experimentation. pH of the aqueous phase used in K_{ow} determination is essential to determination of this parameter. The protonated form is significantly more hydrophilic than the deprotonated form. Since the pKa of 2,4,5-TCP (7.0) is near the pH of water, any small difference in the pH will greatly affect the speciation and will therefore affect the K_{ow}.

Many K_{ow} values have been reported for 2,4-DCP where log K_{ow} values range from 2.90 – 3.23. A smaller variation in K_{ow} values observed for 2,4-DCP than that observed for 2,4,5-TCP. The speciation of ionizable contaminants can have a dramatic effect on partitioning into an organic phase because protonated contaminants are significantly more hydrophobic than are the corresponding unprotonated forms. The decrease in variation observed with 2,4-DCP can possibly be attributed to the higher dissociation constants observed for 2,4-DCP. Because the pKa value for 2,4-DCP is near 8, less variation in ratio of protonated to unprotonated contaminant would be expected when experimental

Table 2.7. Comparison of estimated and literature values of the octanol-water partition coefficient of 2,4,5-TCP.

Log K_{ow}	Source
2.775	LFER from substituted benzenes with polar substituents
4.94	Structure-Fragment Contribution Method
3.72	Leo *et al.,* 1971; Chiou and Freed, 1977; Veith *et al.,* 1979; Bintein and Devillers, 1994; Hansch and Leo, 1979, 1982; Veith *et al.,* 1979; Mackay, 1982; Kaiser, 1983; Kaiser *et al.,* 1984; Veith and Kosian, 1983; Insard and Lambert, 1988; Suntio *et al.,* 1988; Howard, 1991; Veith *et al.,* 1991; Banerjee *et al.,* 1984, 1987; Beltame *et al.,* 1984; McKim *et al.,* 1985; Isnard and Lambert, 1988; THOR, 1989; Commel and Markwell, 1990; Liu *et al.,* 1991; NCASI, 1993 , in Mackay *et al.,* 1985
3.63	Hammers *et al.,* 1982, in Mackay *et al.,* 1985
3.80	Saarikoski and Viluksela, 1982, in Mackay *et al.,* 1985
3.06	Veschueren, 1983, in Mackay *et al.,* 1985
4.19	Schellenberg *et al.,* 1984, in Mackay *et al.,* 1985
4.10	Xie *et al.,* 1984, in Mackay *et al.,* 1985
3.16	Klopman *et al.,* 1985; Pangrekar *et al.,* 1994, in Mackay *et al.,* 1985
3.53	Yoshida *et al.,* 1986, in Mackay *et al.,* 1985
3.88	Xie and Dryssen, 1984; Lagas, 1988, in Mackay *et al.,* 1985
3.84	Beltrame *et al.,* 1988, in Mackay *et al.,* 1985
4.10	Shigeoka *et al.,* 1988; Saito *et al.,* 1993, in Mackay *et al.,* 1985
3.85	Muler and Klein, 1992; Kaiser, 1993; Kolling, 1993, in Mackay *et al.,* 1985
4.02	Kishino Kobayasyi, 1994, in Mackay *et al.,* 1985

data is gathered than for 2,4,5-TCP (pKa = 7.0) because many natural waters and typical toxicity testing medias have a pH near 7.0.

Air-Water Partitioning. Partitioning processes play an important role in the fate of 2,4,5-TCP and 2,4-DCP in the environment. Air-water exchange can be estimated based on the Henry's Law coefficient for a compound, which has been reported for 2,4,5-TCP in the range of 1.30E-3 - 5.82E-3 $atm \cdot L \cdot mol^{-1}$ at 25 °C and 1.08E-3 – 4.29E-3 $atm \cdot L \cdot mol^{-1}$ at 25 °C (Mackay *et al.,* 1985). Lyman *et al.* (1990) state that a Henry's Law coefficient in the range of $10^{-4} – 10^{-2}$ $atm \cdot L \cdot mol^{-1}$ indicates that the substance volatilizes slowly at a rate dependant on K_H, where the gas-phase resistance dominates the liquid phase resistance by a factor of 10 at least. Since the K_H values for 2,4,5-TCP and 2,4-DCP are on the lower end of this range, it can be expected that both compounds volatilize slightly faster than or at the same rate as water, thus they do not preferentially partition into the gas phase, nor do they concentrate in the aqueous phase. Volatilization half-lives for 2,4,5-TCP were estimated to be 9.0 days in a model river 1m deep, flowing 1m/s with a wind speed 3 m/s, and 207 days in a model pond (Howard, 1991). These model estimates, along with consideration of the Henry's Law coefficient, indicate that partitioning into the air is not a major sink for 2,4,5-TCP or 2,4-DCP.

Organic Matter Partitioning. Partitioning into organic matter is an important process for chlorophenols due to their hydrophobic nature. Two useful parameters for quantifying these processes are the organic matter partitioning coefficient, K_{OM}, and the organic carbon partitioning coefficient, K_{OC}. These parameters represent a ratio of a compound

in the organic phase to the compound in the aqueous phase in a manner similar to solvent-water partitioning coefficients. The organic matter-water partition coefficient, K_{OM}, for 2,4,5-TCP is estimated to be 79.25 mol/kg om/mol/Lwater using a LFER based on the aqueous solubility and 575.44 mol/kg om/mol/Lwater using a LFER based on the octanol-water partition coefficient. The log K_{OC} was estimated to be 4.233, using a LFER based on the octanol-water partition coefficient. Literature values for log K_{OC} range from 1.79 – 3.28 (Mackay *et al.,* 1985). Similarly, many values have been reported for the K_{OC} of 2,4,-DCP. Values for log K_{OC} reported range from 1.74 – 3.98 with most typical values being ~2.5. While a wide range of estimates have been found for the organic matter-water partitioning and organic carbon-water partitioning, all estimates indicate that significant amounts of 2,4,5-TCP and 2,4-DCP will partition into the organic phase. This high level of partitioning into organic matter is exemplified in a sorption study performed with soils containing a range of fractions of soil organic matter. Freundlich coefficients for 2,4,5-TCP sorption were found for two soils with 2.08 and 8.36 % SOM, with pH held constant. The reported K_f values were 12.15 and 1.87 respectively, and the reported 1/n values were 0.896 and 0.285, respectively (Frobe *et al.,* 1994). These values show a drastic increase in the quantity of material sorbed with increased soil organic matter quantified by the order of magnitude difference in the K_f values for a 6.28% increase in %SOM.

Acidity is an important parameter for consideration in determining partitioning into organic matter. The fraction of a chemical in the charged phase versus the neutral phase must be considered when analyzing the partitioning or sorption of an ionizable chemical.

It can be expected that the neutral form of 2,4,5-TCP will sorb to organic matter in a manner similar to other nonionic, hydrophobic compounds, while the major mechanism for sorption of the charged species would be dissolution into the organic matter. Thus, a K_{OM} can be determined for both the protonated and deprotonated form of 2,4,5-TCP, with estimates of K_{OM} (TCP^-) being around three orders of magnitude less than that of the K_{OM} (TCP).

Chlorophenol Toxicity

2,4,5-TCP and 2,4-DCP are both irritating to the skin and eyes, with dusts and fumes irritating to the respiratory tract and is readily adsorbed through the skin in toxic amounts. The LD_{50} for toxicity of 2,4,5-TCP to rats is 0.820 mg/kg. This toxicity parameter is higher than that of other compounds in this compound class, with the LD_{50} (rat) of 2,4-DCP equal to 0.58 g/kg and the LD_{50} (rat) of PCP equal to 0.18 g/kg. The 96h LC_{50} for the fish, *Poecilia reticulata* and *Pimephales promelas* are 1.2 and 1,270 μg/L for 2,4,5-TCP and 5.5 and 8.2 mg/L for 2,4-DCP (Verschueren, 1996). Summaries of 2,4,5-TCP and 2,4-DCP toxicity data related to organism of the soils and surface waters, where chlorophenols are often prominent contaminants, is included in Table 2.8 and 2.9 respectively. These data show that 2,4,5-TCP is highly toxic at low concentrations to organisms at all levels of the food chain.

While chlorophenols are toxic in themselves, one of the largest concerns is the formation of dioxins as a by-product of the chlorination of phenol. Typical procedures for the production of 2,4,5-TCP can form 2,3,7,8-tetrachlorodibenzo-p-dioxin which poses a

Table 2.8. 2,4,5-TCP toxicity data (from Mackay *et al.,* 1985).

Toxic Threshold (mg/L)	Toxicity Indicator	Organism Type	Organism
8.3	EC_{50}	Microorganisms	A.S. respiration inhibition
28	EC_{50}		Biodegradation inhibition
1.5	Toxicity observed	Algae	*Chlorella pyrenoidosa*
0.68	24h EC_{50}		*Tetrahymena pyriformis*
0.49	IC_{10}		*Scenedesmus subspicatus*
1.1	IC_{10}		*Scenedesmus subspicatus*
3.5	7d LC_{50}	Crustaceans	*Daphnia magna*
1.5, 2.1	24h EC_{50}		
0.9, 1.0, 2.7	48h EC_{50}		
0.0038-0.0056	14d LC_{50}	Earthworms	*Eisenia Andrei*
0.002-0.038	14d LC_{50}		*Lumbricus rubellus*
1.9	48h LC_{50}		Tubifex
1.7	24h LC_{50}	Fish	Goldfish
1.0	48h LC_0		*Idus idus melanotus*
1.3	48h LC_{50}		
1.6	48h LC_{100}		
2.5	48h LC_{50}		*Oryzias latipes*
1.270	96h LC_{50}		*Pimephales promelas*
0.0012	96h LC_{50}		*Poecilia reticulate*
0.74	>7d LC_{50}		

Table 2.9. 2,4-DCP toxicity data (from Mackay *et al.,* 1985).

Toxic Threshold (mg/L)	Toxicity Indicator	Organism Type	Organism
105	EC_{50}	Microorganisms	A.S. respiration inhibition
44	EC_{50}		Biodegradation inhibition
<1	EC_{50}		*Pseudomonas pictorum*
4	EC_{50}	Algae	*Chlorella pyrenoidosa*
3.6	7d EC_0		*Scenedesmus quadricauda*
2	8d EC_0		*Microcystis aeruginosa*
2.5, 2.7, 2.8, 3.9	24h EC_{50}	Crustaceans	*Daphnia magna*
1.3, 1.4, 2.6	48h EC_{50}		
0.063-0.083	14d LC_{50}	Earthworms	*Eisenia Andrei*
0.104-0.263	14d LC_{50}		*Lumbricus rubellus*
7.8	24h LC_{50}	Fish	Goldfish
8.2	96h LC_{50}		Pimephales promelas
5.5	96h LC_{50}		Poecilia reticulate
4.2	>7d LC_{50}		

serious concern to human health (Mark *et al.,* 1978). Dioxins are also a by-product of the combustion of chlorophenols, making the incineration of chlorophenol containing material a health concern.

Environmental Persistence of Chlorophenols

Photolytic, Photooxidative and Hydrolytic Degradation. Photolytic degradation plays an important role in chlorophenol persistence in the environment. While the photodegradation pathway for chlorophenols has not been detailed due to the unstable nature of products formed by radical attack on the phenolic ring, this transformation reaction is considered to be the major sink for chlorophenols in the environment. Several ring-opened products have been identified from decomposition of chlorophenol including maleic, oxalic, acetic and formic acids, with ultimate products being CO_2, H_2O and Cl^- (Androulaki *et al.,* 2000). Chlorination of the phenol ring increases the rate of photolytic decomposition, with chlorine in the ortho position having the least pronounced effect on transformation rate (Androulaki *et al.,* 2000). Aquatic photolysis half lives are given as 0.5 hr and 336 hr (2,4,5-TCP) and 0.8 hr and 3 hr (2,4-DCP) under summer and winter sunlight conditions at 25 and 18°C, respectively (Howard *et al.,* 1991). Photooxidation plays a less significant role in chlorophenol degradation, with half-lives in water reported as 66 - 3480 hr (2,4,5-TCP) and in air as 30.1 – 301 hr (Howard *et al.,* 1991). Hydrolysis is considered to play virtually no role in 2,4,5-TCP degradation with a first-order hydrolysis of $>8x10^6$ years (Howard, 1991).

Microbial Degradation. Microbial degradation plays a role in the decomposition of 2,4,5-TCP, particularly under conditions without significant light penetration such as in soils and sediments. Jin and Bhattachary (1997) found degradation of 2,4,5-TCP, forming 3,4-dichlorophenol, 3-chlorophenol and phenol in an incubation with anaerobic sludge. The same daughter products were isolated in an incubation of 2,4,5-TCP in a methanogenic culture with sewage sludge as the microbial source (Madsen and Aamand, *et al.,* 1992). 4-Chlorophenol was found to be the primary degradation product of 2,4-dichlorophenol in freshwater sediment, anaerobic freshwater sediment, methanogenic sediment and anaerobic sludge (Kohring *et al.,* 1989; Zhang and Wiegel, 1990; Ukin *et al.,* and Jin and Bhattachary, 1997). Chang (2000) showed that 2,3,4,6-tetrachlorophenol can be completely degraded by methanogenic cultures through reductive dechlorination with plant-derived organic matter as an electron donor. Therefore, chlorophenols have been shown to be degraded under aerobic and anaerobic conditions. Anaerobic degradation proceeds through reductive dehalogenation and is typically more favorable for the highly chlorinated phenols (eg., Rittman and McCarty, 2001; Sanford and Teidje, 1997; Cozza and Woods, 1992; Fetzner *et al.,* 1998). Aerobic degradation occurs more readily with less-chlorinated phenols than highly chlorinated phenols and typically proceeds through oxygenolytic dehalogenation (i.e., introduction of a hydroxyl group in the place of a chlorine), although pathways for hydrolysis and other fortuitous reactions exist (Rittman and McCarty, 2001; McAllishter *et al.,* 1996). Microorganisms capable of aerobic dechlorination can typically gain carbon and energy from oxidation and mineralization of chlorinated phenols. In addition, several strains of *Desulfitobacterium* species can use chlorinated compounds as electron acceptors under anaerobic conditions

and have the ability to dechlorinate chlorinated phenols, implying that chlorinated phenols can be used as an electron acceptor (Utkin *et al.,* 1995; Sanford et al, 1996; Löffler *et al.,* 2003).

A wide range of half-lives has been reported for microbial degradation of chlorophenols. Values ranging from 552–16560 hr (2,4,5-TCP) and 66.7-199 hr (2,4-DCP) have been reported for aerobic biodegradation. Values ranging from 3028–43690 hr (2,4,5-TCP) and 324-1032 hr (2,4-DCP) were reported for degradation under anaerobic conditions (Howard *et al.,* 1991). Other reports for degradation of 2,4,5-TCP include $t_{1/2}$ of 16560 hr for degradation in Skidway River water, and 552 hr in water-sediment slurry and a 690 days in river water (Mackay *et al.,* 1985). Additional reports of 2,4-DCP degradation include that of negligible degradation in total darkness and complete degradation within 16 – 23 days (Howard *et al.,* 1991). Degradation rates of $1.43x10^{-20}$ mol•cell^{-1}•h^{-1} (2,4,5-TCP) and $3.76x10^{-19}$ mol•cell^{-1}•h^{-1} (2,4-DCP) were reported from studies with pure cultures of degrading organisms, and $5x10^{-15}$ mol•cell^{-1}•h^{-1} (2,4,5-TCP) and $0.2\text{-}2x10^{-14}$ mol•cell^{-1}•h^{-1} (2,4-DCP) were reported with microorganisms isolated from the Seneca River (Mackay *et al.,* 1985).

Fluorinated Analogs of Chlorophenols

Fluorinated analogs of chlorophenols were used track compound fate internal to plants using ^{19}F NMR. This section includes a description of how replacing a chlorine substituent with a fluorine substituent in phenolic compounds affects physical and chemical properties, compound stability and fate in the environment. Linear free energy

relationships (LFER) and methods for estimating using 2,4-dichlorophenol and 4-chloro-2-fluorophenol as a model compound

.

Physical and Chemical Property Variation. Small variations are observed in the chemical and physical properties of 2,4-DCP when the ortho chlorine is replace with fluorine to create 4-chloro-2-fluorophenol. Fluorine is more electronegative than chlorine (F: 4.0; Cl: 3.0) thus conferring a stronger polarity to the carbon-fluorine bond than the carbon-chlorine bond (Key *et al.,* 1997). Carbon-fluorine bonds have one of the strongest bond energies in nature, a contributor to the stability of many organofluorines.

A comparison of environmentally relevant chemical and physical properties for 2,4-DCP and 4-chloro-2-fluorophenol is included in Table 2.10. A fluorine in the ortho position increases aqueous solubility and vapor pressure, an effect that causes the K_H to remain somewhat static. Thus 4-chloro-2-fluorophenol is expected to partition into air at roughly at the same rate as water, i.e., it does not preferentially partition into the gas phase, nor does it concentrate in the aqueous phase, behaving similarly to 2,4-DCP. The substitution of a fluorine increases the pK_a from 7.9 to 8.3, a value at which speciation of the contaminant will be important to its behavior in environmental systems. By inspection of K_{ow} values it is clear that the hydrophobicity of the fluorinated analog is decreased compared to 2,4-DCP, however 4-chloro-2-fluorophenol retains some degree of hydrophobicity with a log K_{ow} of 2.33 – 2.66. 4-Chloro-2-fluorophenol and 2,4-DCP can be expected to behave similarly in both air-water partitioning and organic matter partitioning.

Table 2.10. Comparison of physical and chemical properties for fluorinated analogs.

Property	2,4-dichlorophenol	4-chloro-2-fluorophenol	Data Source for Fluorine Substituted Compound
Vapor Pressure	1.58E-4 – 7.8E-5 atm	5.45E-4 atm	LFER*
K_H	1.08E-3 – 4.29E-3 atm L mol^{-1}	1.39E-3 atm L mol^{-1}	LFER*
pK_a	7.9	8.3	Hammett Correlation
Water Solubility	4.47-6.19 g/L	9.82 g/L	LFER*
log K_{ow}	2.90 – 3.23	2.33 – 2.66	Fragments and Factors (Lyman *et al.,* 1982)

* LFER modeled after work presented in Schwarzenbach *et al.,* 1993

Biological Activity of Fluorinated Organics. Fluorinated organics have received less attention in terms of fate in the environment because they are generally considered to be more biologically inert and less likely to have an impact on human health than their chlorinated and brominated counterparts. However, many fluorinated organics have been shown to exhibit toxicity through inhibition of enzymes, cell-cell communication, membrane transport and energy production and initial research on production and toxicity of fluorinated organic materials stemmed from military-related interests in neurotoxins (Ciba Foundation, 1971; Key *et al.,* 1997). Degradation of fluorinated compounds has been studied in natural systems. Dehalogenation reactions have been studied by numerous researchers and it has been demonstrated that enzymatic processes readily dehalogenate haloorganics. Several enzymes known to dehalogenate chloroorganics, bromoorganics or iodoorganics cannot act on fluoroorganics (Neidleman and Geigert, 1986). Other enzymes responsible for dehalogenation, such as some haloacetate delahgenases in *Pseudomonas* sp. and *Fusarium solani*, show increased specificity for fluorine over other halogens (Neidleman and Geigert, 1986). Biologically mediated reductive, hydrolytic and oxidative defluorination have been observed for fluorinated organics (Key *et al.,* 1997). Researchers have also observed conjugation of fluorinated compounds with cysteine and glucoronic acid in rats and mice (Key *et al.,* 1997). Some oxidative enzymes responsible for dehalogenation, such as peroxidases and oxygenases, have been shown to have low substrate specificity and do not distinguish between the halogens (Neidleman and Geigert, 1986). Alternative means of defluorination involves attack at functional groups on adjacent moieties, where further reaction will result in decarboxylation, desulfonation, deamination and fluorine elimination (Key *et al.,* 1997).

Fluorinated aromatics are widely used (e.g., estimated world market in 1994 was 10,000 t). Fluoroaromatics have been shown to be attacked oxidativley yielding transformation products with variable defluorination and toxicity. The degradation of diflubenzuron has been demonstrated by several fungal isolates, however degradation products persist in the environment and are toxic to aquatic organisms and soil microbes. Bacterial isolates have been shown to degrade fluoroaromatics through dioxygenase attack and hydrolytic defluorination (Key *et al.,* 1997).

Degradation, conjugation and defluorination has been demonstrated for fluorinated organic compounds, however, enzyme specificity for fluorinated compounds is not identical to that of chlorinated compounds. Thus, use of fluorinated analogs in biological systems could be expected to closely parallel a chlorinated molecule in reactions with non-specific enzymes and in reactions with adjacent sites on the molecule.

Fluorine in Biological Systems. Fluorine does not perform a vital function in animals or plants and is not considered dietary essential (National Research Council (U.S.) Committee on Biological Effects of Atmospheric Pollutants, 1971). Little background fluorine is present in plants and animals as fluoride does not accumulate in plant or animal systems (Ratcliffe and Roscher, 1998). Only around twelve naturally occurring fluorinated organics have been identified and little is known about the biochemistry of fluorination (O'Hagan and Harper, 1999). Fluoroacetate has been shown to accumulate in certain semi-arid plants, and several of the fluoroacetate accumulators have also been

shown to contain a small quantity of fluorocitrate (O'Hagan and Harper, 1999; Neidleman and Geigert, 1986). Compounds such as fluorinated fatty acids and fluoroacetone are also produced by several semi-arid plants. Microorganisms also produce fluorinated organics, with isolated examples including nucleocidin, 4-fluorothreonine and fluoroacetate. Many of the biologically produced fluoroorganics are acute poison and neurotoxins and are likely produced by plants and microorganisms for protection against predation (O'Hagan and Harper, 1999).

Nuclear Magnetic Resonance

Analytical Overview

Nuclear Magnetic Resonance (NMR) is an analytical technique that reveals useful information about the chemical structure of a molecule. When a spinning nucleus is placed in a magnetic field, the spin orients itself parallel or antiparallel to the magnetic field with parallel orientation dominating because it is the lower energy state. Upon irradiation by an electromagnetic field, a nucleus will adsorb photons from the electromagnetic field causing the nucleus to transition between energy states. The number of photons adsorbed can be detected and produces a resonance line. Advantageously, each isotope with a nuclear spin adsorbs photons at a unique frequency ensuring that a researcher encounters absorption peaks from only one nucleus in a NMR spectrum. The presence of an electron cloud surrounding a nucleus manifests itself as a shift in the resonance line. Thus, a change in the magnetic shielding of a nucleus caused by different molecular surroundings causes a shift of resonance line (chemical shift). The

chemical shift and intensity of an NMR spectrum can be used to identify and quantify compounds thus establishing NMR as a technique that allows for identification of modifications in molecules and quantification of those modified molecules (Veeman, 1997; Rietjens, 1993).

NMR has been by chemists to determine structures of synthetic materials and biologists for elucidation of three dimensional structures of macromolecules such as proteins and carbohydrates. Medicine has widely used NMR imaging to non-invasively study spatial distribution and kinetics of pharmaceuticals and their metabolites internal to human and animal subjects (Wolf *et al.,* 1990). More recently, NMR has been recognized as a valuable tool for metabolite identification by environmental engineers and food scientists. In particular, fluorine NMR (^{19}F NMR) has been found to be especially valuable in metabolite identification because it is a highly sensitive nucleus (83.3% of the sensitivity of ^{1}H). Another advantage is that ^{19}F NMR has a broad chemical shift range due to the fact that ^{19}F is highly sensitive to its molecular environment (500 ppm range for ^{19}F versus 15 ppm for ^{1}H and 250 ppm for ^{13}C). This broad chemical shift range reduces the chance of peak overlap and produces a different signal for molecules that are modified several bond distances away from the fluorine nucleus. Everett *et al.* (1985) demonstrated this feature of the fluorine nucleus through examination of flucloxacilin where four distinct peaks were detected for flucloxacilin and its metabolites even though the modification of flucloxacilin is several bond distances away from position of the fluorine (Figure 2.5). Another advantage of fluorine is the absence of background signals since biological systems do not contain NMR visible fluorinated compounds

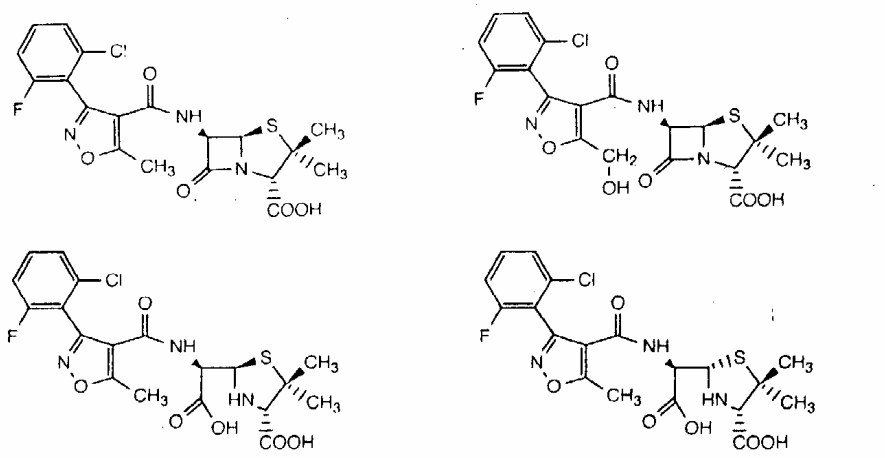


Figure 2.5. Flucloxacilin and three metabolites identified by four distinct peaks on the ^{19}F-NMR spectra. Note the distance on the molecule between the fluorine substituent and the modification (from Everett *et al.,* 1985 in Rietjens *et al.,* 1993).

(Rietjens *et al.,* 1993). This implies that all resonances observed can be attributed to the fluorinated compound of interest.

Identification and Detection of Metabolites

^{19}F NMR has been used to reveal degradation and transformation products of several fluorinated compounds in microbial systems. The degradation pathways of fluorinated phenols by several species of bacteria were investigated *in vivo* using liquid-phase ^{19}F-NMR to identify fluorocatechols, fluoromuconates and the fluoride anion (Boersma *et al.,* 1998; Bondar *et al.,* 1998). The ^{19}F NMR analyses were performed on centrifuge supernatant liquid from cell cultures incubated with various fluorophenols. Identification of metabolites was accomplished through comparison with prepared standards and quantification was achieved through peak area comparison with a known internal standard. Advantages of investigating fluorinated phenol degradation pathways observed in these studies include easy quantification, absence of background signals, and the benefit of quantifying fluoride anion using the same technique as was used for quantification of other products. Additionally, no metabolite extraction or separation steps were required, thus unstable compounds could be examined without risk of further transformation. A similar study investigated the biological, thermal and photochemical degradation pathways of 2-trifluoromethylphenol (Reinscheid *et al.,* 1998). This study coupled ^{19}F NMR with corresponding mass spectrum data, thus determining the structure and formation kinetics of metabolic products. Other related studies include investigation of biotransformation pathways and regioselectivity of specific aromatic degradation enzymes (Peelen *et al.,* 1995; Cass *et al.,* 1987).

^{19}F-NMR has also been used in plant and animal systems to elucidate metabolites derived from pollutants and pharmaceuticals. These more complicated applications use two related techniques for NMR analysis: detection of metabolites once extracted from plant systems (liquid phase NMR) and detection of parent compounds and metabolites *in situ* (solid state NMR). For example, Serre *et al.* (1997) investigated the degradation of a fluorinated fungicide N-ethyl-N-methyl-4-(trifluoromethyl)-2-(3,4-dimethyloxyphenyl)benzamide in plant cells. These researchers analyzed solvent extraction supernatant using ^{19}F NMR to identify and quantify metabolites paired with radiolabled carbon analyses to determine what fraction of material was removed from the plant by solvents. ^{19}F NMR was a sensitive multiresidue analysis method, giving direct identification of derivatives in plant material. Another study used solid state NMR to track uptake of trifluoroacetic acid (TFA) by hydroponic tomato plants (Rollins *et al.,* 1989). This study used an external probe placed around the plant leaf to demonstrate that TFA was continuously taken up by the plant and no metabolic products were formed within plants. NMR imaging using the ^{19}F nucleus was also used to provide a two-dimensional description of distribution of TFA within the plant leaf.

Several studies tracked behavior of fluorinated compounds in animals using techniques similar to those used in plant and microbial systems. Researchers identified metabolites of pharmaceuticals and contaminants in bodily fluids (e.g., blood, urine) of rats using fluorinated analogs (Rietjens *et al.,* 1993). Researchers used ^{19}F NMR to monitor the quantity of Ca^{2+} having perfused into intact ferret hearts under physiological conditions

by using a fluorine labeled Ca^{2+} chelator (Marban *et al.,* 1987). The analysis involved excising the heart from a treated ferret, exposing the heart to fluorinated chelator and analyzing the entire heart using solid state ^{19}F NMR. The chelator gave separate signals depending on if it was bound to or free of Ca^{2+} allowing researchers to determine the quantity of free Ca^{2+} present. Another study looked at the *in vitro* behavior of proinsecticides fluorinated carboxylic acids *in vitro* in insect tissues using ^{19}F NMR to monitor the concentration of the fluorinated compounds internal to insect tissues (Hamm *et al.,* 1999).

CHAPTER 3

MATERIALS AND METHODS

Methodologies used for experimental studies detailed herein are described providing general procedures and parameters used. All chemicals used in this study were the best grade commercially available.

Experimental Reactor Protocol

A fixed mass of *L. minor* was exposed to contaminants in unsealed 145-mL serum bottles or 500 mL Erlenmeyer flasks. All plant mass units provided are fresh weight and all water used was deionized water. Contaminants were dissolved in a fixed volume of modified duckweed nutrient media in accordance with Method 8211D (Greenberg *et al.,* 1998) at a range of concentrations significantly below solubility in water (Howard and Meylan, 1997). Modifications of 4 mM of total P (K_2HPO_4 or KH_2PO_4) and 2 mM of total C ($NaHCO_3$) and substitution of sulfate salts for chloride salts were made to increase buffer capacity and provide a low chloride-ion background. Small volumes of NaOH or H_2SO_4 were used to titrate media to desired pH values (pH was a value of 5 – 9 for experiments described herein). The duckweed nutrient media, modified as described above and titrated to a specified pH will hereafter be referred to as media. An example profile of pH of media with time for plant exposure to TCP is presented in Figure 3.1 for systems titrated to initial pH values of 6 and 8. Data demonstrated that pH values did not

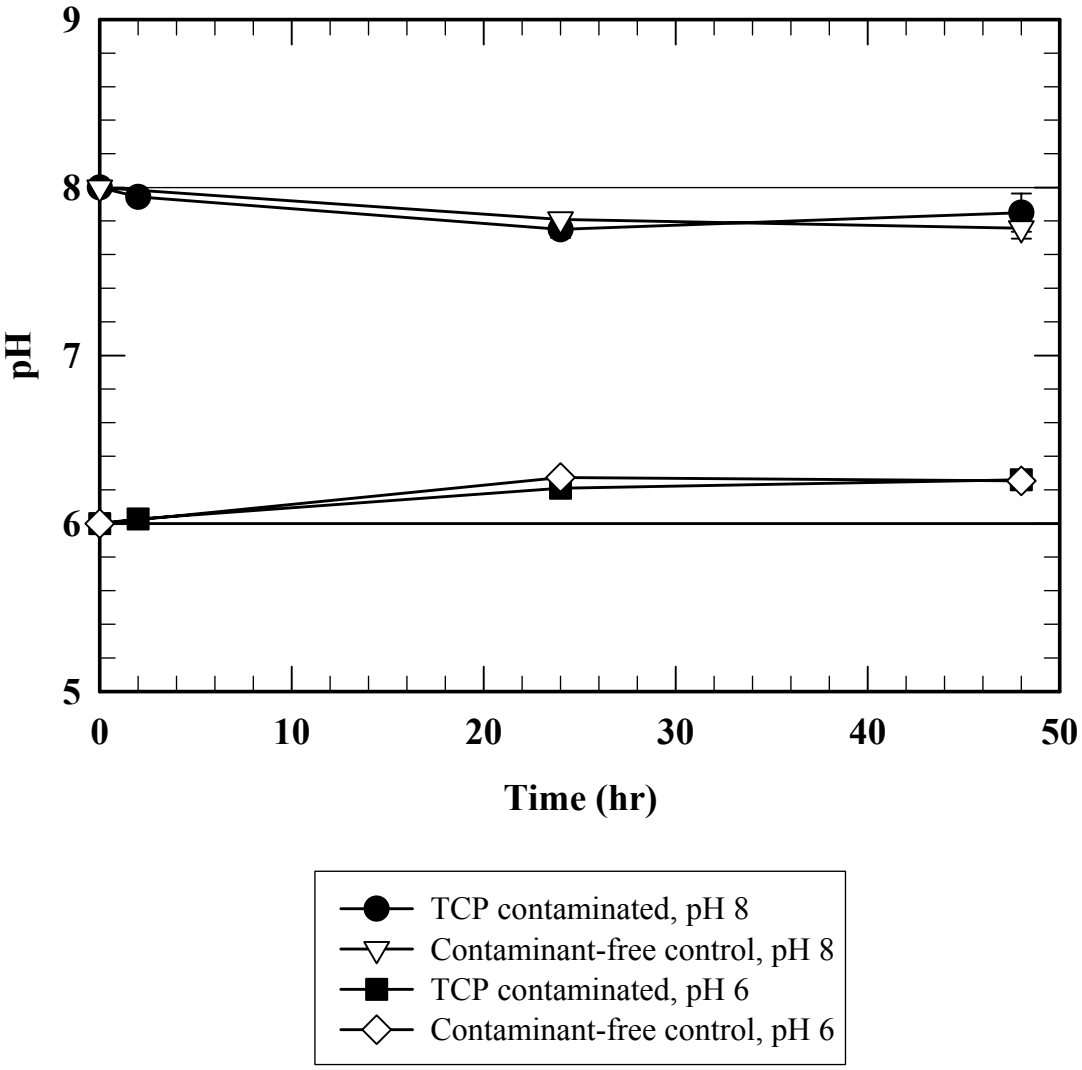


Figure 3.1. Example pH profiles with time for *L. minor* exposed to TCP at pH values of 6 and 8.

significantly vary from initial pH values and the buffer used effectively fixed pH values in experimental systems described herein. *Lemna* sp. have the ability to grow readily at media in the pH range of 3.5-10.5 (Cleuvers and Ratte, 2002) and intracellular pH has been shown to be independent of external pH for the external pH range of 4.5 to 7.5 in *Acer speudoplatanus* cell suspension cultures (Gout *et al.,* 1992).

Contaminant addition to media was achieved by diluting a stock solution of halogenated phenol (prepared in <2% methanol to enhance solubility) with DI water to achieve desired concentrations, where a dilution greater that 1:1000 was typically used. All media was mixed in bulk to minimize variation within an experiment and split for triplicate reactors. A matrix detailing experimental parameters for results presented herein is displayed in Table 3.1. Plants and contaminants examined are listed along with plant densities and contaminant concentrations used. Parameters describing reactor size, media volume, and mass of plants used are provided. Plant density (g FW plant / media volume) was an important parameter throughout experiments described herein, where increasing plant density often increased rate of contaminant uptake and decreased toxic effects. The density of *L. minor* used always provided full coverage of media surface, where it was estimated that ~15% of the surface was not covered by plants in any system as a result of natural gaps between fronds. *M. aquaticum* systems did not provide significant coverage of media surface because of the emergent nature of the plant.

Table 3.1. Experimental series detailed herein with corresponding contaminants examined, plants used and experimental parameters used. The chapter where experimental results are described is listed.

Experimental Series	Contaminants Examined	Plant Examined	Concentration Range (μM)	Reactor Volume (mL)	Aqueous Volume (mL)	Plant Mass (g)	Plant Density (g/L)	Data Location
Chlorophenol Uptake	2,4,5-TCP 2,4-DCP	*L. minor*	2.3 - 66.3	145	130	2	15	Chapter 4 Chapter 6 – 7
	2,4,5-TCP	*M. aquaticum*	5.1 - 253.2	1000	500	25	50	
Halogenated Phenol Uptake	2,4,5-TCP 2,4,6-TCP 2,3,5-TCP 2-Cl-3,5-DFP 2,3,5-TFP 2,4-DCP 2,6-DCP 4-Cl-2-FP 2-Cl-4-FP 3,5-DFP 2,4,6-TBP 2,3,4,6-TeCP	*L. minor*	2.3 - 25.7	145	100	1	10	Chapter 4
Contaminant Sequestration (^{14}C)	2,4,5-TCP 2,4-DCP	*L. minor*	13.7 12.3	450	200	5	25	Chapter 5
	2,4,5-TCP	*M. aquaticum*	22.3	450	200	15	75	
NMR	4-Cl-2-FP	*L. minor*	50.6 – 96.2	450	200	4.5	22.5	Chapter 5

Details of plant sequestration of 2,4-DCP for investigation of microbial degradation of sequestered contaminants are provided with results in Chapter 8. During experimental studies, plant reactors were continuously illuminated under cool white light, with temperature held constant at 22° C in a climate-controlled room. Exposure reactors were fitted with aluminum-foil shields designed to allow light to illuminate surfaces of plants, and block light penetration through reactor side-walls to minimize photolytic reactions and algal growth. Sacrificial sampling was used for experiments which required further use of plants for oxygen production rate, NMR measurements or ^{14}C analyses. Aqueous phase samples were taken by removing media with a plastic syringe and blunt-ended pipette for all kinetic data gathered where sacrificing reactors was unnecessary. Data were gathered on triplicate reactors for all experimental series with the exception of NMR analyses which were single sacrificed reactors.

Inactivated-plant experimentation followed the same protocol as live plant experimentation, using plants exposed to sodium azide solution (1g/L) for 5 - 7 d before experiments were initiated. Oxygen production rate measurements for representative experiments verified photosynthetic production had ceased after sodium azide pretreatment. Inactivated plants were subsequently treated identically as live plants in contaminant exposures.

Oxygen Production Rate Measurements

Oxygen production rate was measured to gauge plant activity and establish relative inhibition due to contaminant exposure. Sacrificial sampling was used and all systems

were run in triplicate. Plants were exposed to contaminant for a designated time, after which reactors were sacrificed and plants were used in oxygen production rate measurements. During a sampling event, plants were separated from media using a screened scoop and pH of residual media was measured and an aqueous sample was taken for halogenated-phenol analysis. To measure oxygen production rates, plants were split evenly on a mass basis into two reactors which contained 100 mL of TCP-free media (pH 7). Reactors were fitted with Teflon-coated, butyl-rubber septa. One reactor was completely covered with aluminum foil, blocking all light, and the other reactor was left uncovered.

A variable-headspace, graduated, U-tube manometer was used to allow production or consumption of carbon dioxide and oxygen without significant deviations from atmospheric pressure and to identify total gaseous volume. A needle connected one end of the manometer to the sealed reactor and the other end of the manometer was open to the atmosphere. The reactor headspace volume and volume in tube were measured by volume displacement, therefore changes in manometer level were used to calculate total headspace volume. Manometer fluid (Method 2720B; Greenberg *et al.,* 1998) was designed to avoid oxygen and carbon dioxide scavenging. After a set period of time (*e.g.,* 20 hr), total gaseous volume for each reactor (total gaseous volume = reactor headspace + tube volume +volume in manometer) and atmospheric pressure were recorded and partial pressures of major headspace gases were assessed via GC-TCD. Total moles of oxygen in the reactor were calculated using measured partial pressure of oxygen, total headspace volume, temperature and pressure. The dark reactor represented

oxygen consumption by microbes and plants, and parallel consumption was assumed to occur in the light reactor. Example oxygen data for no-TCP exposed system and a TCP exposed system (3.57 mg/L) are presented in Figures 3.2 and 3.3, respectively. Data points represent reactors sacrificed after 0, 24 and 48 hr of exposure and show total moles oxygen in light and dark bottles produced for 20 hr after reactor sacrifice. Each data point shown represents measurement from triplicate reactors, and each light reactor measurement had a corresponding dark reactor measurement. In control systems (e.g., Figure 3.2) total mols produced in light exposed reactors and dark reactors were constant with exposure duration. Inhibited systems (e.g., Figure 3.3) experienced a small increase in oxygen content in dark reactors after increased exposure duration, however oxygen content in light exposed bottles decreased significantly with exposure duration in inhibited systems. Total moles produced by plants were determined by subtracting total moles in the dark reactor from total moles in the light reactor. Total moles produced by the plant were normalized to test duration (typically 20 – 24 hr) giving Oxygen Production Rate, which has been defined using the term α.

^{14}C Studies

Experimental reactors consisted of 0.5 L Erlenmeyer flasks fitted with a rubber stopper. Rubber stoppers were fitted with two stainless-steel pipettes, one long enough to reach the bottom of the reactor for aqueous sample extraction and one extending 10 cm into the reactor to facilitate headspace evacuation. Each pipette had a plastic stop-cock attached to maintain a closed headspace during an experimental run and to regulate headspace gases during media and headspace evacuation. Media was titrated to pH 8 using 6N HCl,

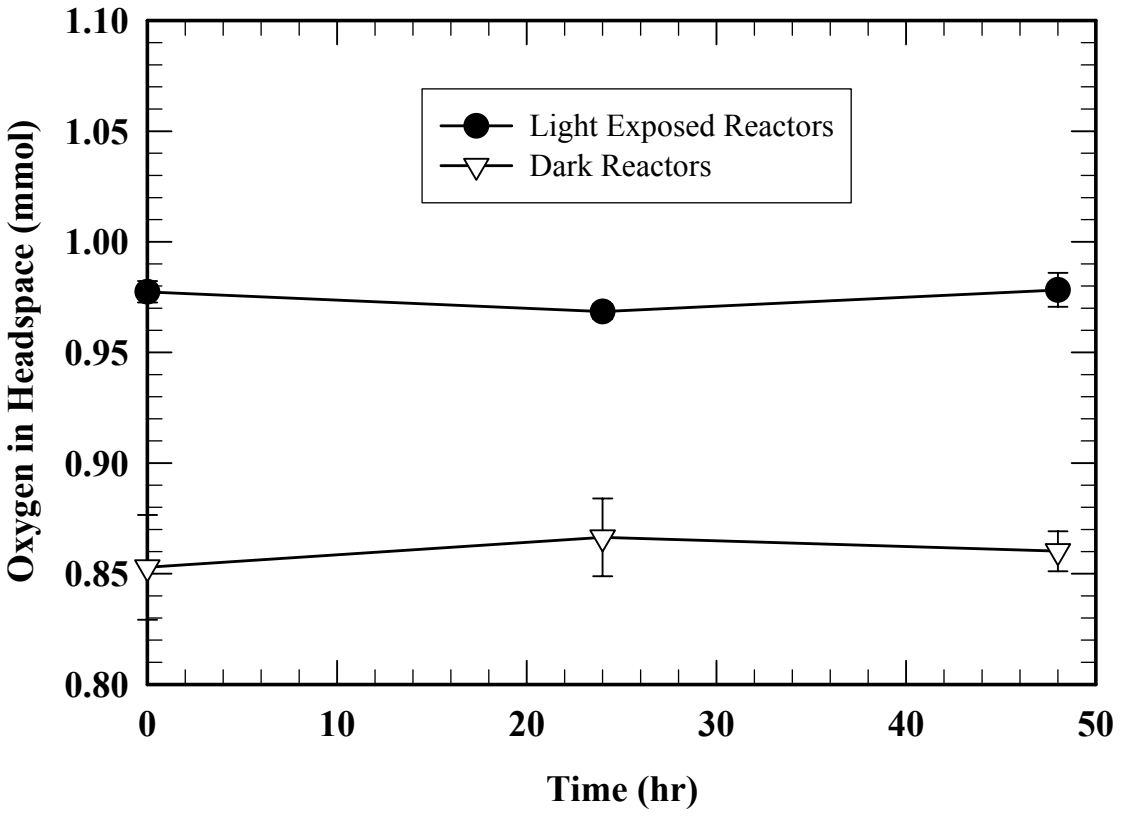


Figure 3.2. Oxygen produced in light exposed and dark reactors in an uninhibited system which was not exposed to contaminant.

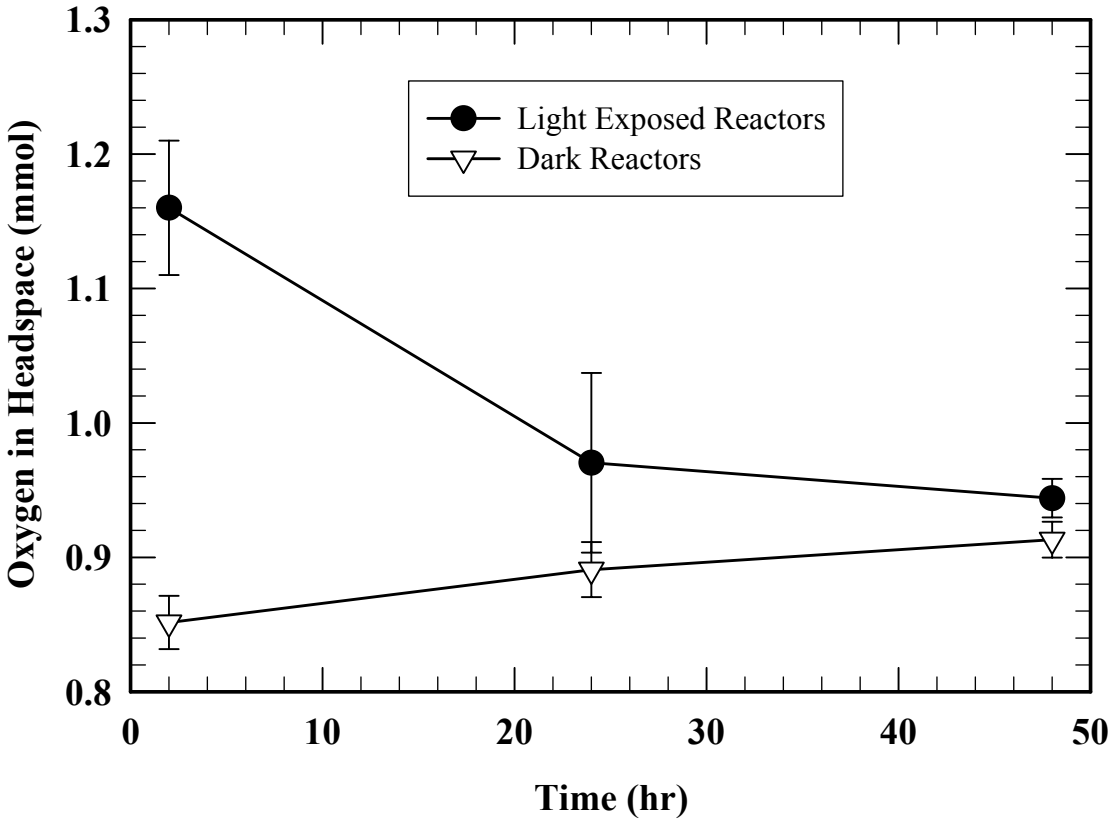


Figure 3.3. Oxygen produced in light exposed and dark reactors in a system exposed to 3.79 mg/L 2,4,5-TCP at pH of 6.

and ^{14}C amended reactors (radiolabeled reactors) were run in parallel with reactors with no ^{14}C (unlabeled reactors). A relatively small volume (~50 μL) of ^{14}C labeled contaminant stock solution (^{14}C labeled 2,4,5,-TCP or ^{14}C labeled 2,4-DCP) was added to media in radiolabeled reactors, where ^{14}C-labeled material comprised <0.001 – 0.06% of total contaminant in radiolabeled reactors. Total ^{14}C activity in radiolabeled reactors ranged from 0.0128 – 0.1896 μCi. Processes occurring in radiolabeled and unlabeled reactors were identical, therefore, HPLC-MS analyses were only run on samples taken from unlabeled reactors.

Reactors containing a recorded mass of media (200 mL) were spiked with a small quantity of an aqueous solution of ^{14}C tracer depending on desired level of radiation in reactor. The media and contaminant tracer were quickly homogenized and an initial sample of 6 mL was taken. Next, the plants were added to the reactor and the reactor was sealed.

To assess the fate of the ^{14}C-tracer after a given time, media was extracted from the reactor via the sampling syringe, where the void volume was refilled with N_2 gas. A small quantity of media (~10 mL) could not efficiently be removed from reactor using the syringe due to plant fouling of the pipette and the mass of media remaining was calculated by weight difference. A positive pressure of N_2 gas was used to evacuate the head space for 20 min through two gas tight traps in series. The headspace traps contained 10 mL R. J. Harvey trapping solution and were designed to capture all gaseous organic or inorganic carbon. The second trap never contained any radioactive material,

thus it was assumed that all headspace ^{14}C was caught in the first trap. Plants were removed from the reactor and rinsed with 20 mL water saving plant rinseate. Plants were blotted dry and massed. Reactors were rinsed with deionized water, and rinse water was collected with plant rinse water, where total rinse volume was measured. The ^{14}C contained in rinse was calculated as total activity in rinse water less the activity of media which remained in reactor when the bulk of the media was evacuated through the pipette.

Radioactive materials contained in the plant material were quantified using a R. J. Harvey Biological Oxidizer. Ground plant material were combusted in 1 g aliquots at 900 °C with the input of pure O_2 for four minutes as a carrier gas, where CO_2, the product of combustion was caught in 15 mL of R. J. Harvey trapping solution. Triplicate samples of plant material were oxidized for each reactor, and the average relative standard deviation (RSD) of plant oxidation measurements was 6.8%. ^{14}C standards (chlorophenol or bicarbonate) were oxidized in triplicate to calculate the oxidation recovery. Oxidation recovery ranged from 81 – 99%, and a correction factor was applied to plant oxidation measurements. Radioactive materials were quantified via liquid scintillation on a Beckman LS II. Triplicate liquid scintillation measurements of a standard sample produced RSD values >2%.

Analytical Methods

HPLC

Aqueous samples were filtered through a 0.2 mm PTFE syringe filter, and acidified before analysis with an addition of glacial acetic acid equal to 5% of the original sample

volume. Substituted phenol concentration was quantified via HP 1100 Liquid Chromatograph with a diode array UV-Vis detector and a mass selective detector (LC-MS). Calibration curves containing four or more data points were generated with each sample run and R^2 values for curves were always greater than 0.99. Periodically, a standard sample was injected repeatedly to verify instrument precision with results generating RSD values which were typically less than 1% and ranged from 0.2 – 2.1%. In addition, standards were interspersed throughout sequences to verify instrument precision, performance, and reliability. Standard recoveries during experimental sequences ranged from 96.8 – 99.2%.

GC-TCD

Headspace gases were analyzed using a HP 5890 gas chromatograph equipped with a thermal conductivity detector (GC-TCD). A 50 μL headspace sample was analyzed on a Pora PLOT Q, fused silica column to determine oxygen content, followed by a 500 μL injection analyzed on a Moesieva 5A, fused silica column for determination of carbon dioxide partial pressure. As an example of sample measurement repeatability, triplicate measurements of partial pressure of O_2 in the headspace of control reactors generated RSD values that ranged from 0.2 – 1.4%.

Nuclear Magnetic Resonance

Sample Preparation. After plant exposure to fluorinated contaminant, reactors were sacrificed and plants were split for use in oxygen production rate measurements (2 g) with remaining plant material frozen at -80°C and reserved for later use in NMR

experimentation. To prepare liquid NMR samples, plants were removed from -80°C freezer and macerated with mortar and pestle until a find powder was achieved. A given mass of powdered-plant was placed in a test tube with 3 mL of acetonitrile and 100 µL 5% o-phosphoric acid. Samples were shaken by hand for 5 min and allowed to sit for 30 min after which samples were centrifuged at 3000 rpm for 10 min. Supernatant was removed and the extraction was repeated three times. Extract supernatant was added to each NMR tube and the volume added was calculated by measuring length of sample in NMR tube and the tube diameter specifications provided by the manufacturer. A known concentration of an internal standard (e.g., fluorobenzene, 2,6-dichloro-4-fluorophenol) was mixed in dueterated acetonitrile. A small, known mass of internal standard was added to the sample in the NMR tube using weight differential and density of acetonitrile to calculate volume of standard added (typically ~ 1 drop added to each NMR sample). The concentration of internal standard was targeted to be near that of the expected concentrations of parent material and metabolites in plant extracts.

Instrumentation Used. Instrumentation for NMR assessments was located at Georgia Tech and Emory University. The primary instrumentation used for liquid phase assessment of fluorinated metabolites was located at Emory University and was an INOVA 400, equipped with a 9.4 T superconducting magnet. The instrument contained a auto-tune probe capable of detecting ^{1}H, ^{13}C, ^{19}F and ^{32}P, and was manually shimmed to achieve optimal peak shape. Number of scans used in analyte detection was unique for each sample or group of samples depending on expected fluorine concentration.

Plant Source and Stock Cultures

Plants were maintained in an outdoor, simulated lagoon composed of six, 200-L containers fed with 500 g of Miracle Gro plant fertilizer monthly. Many of the containers in the simulated lagoon held pots containing natural soils planted with rooted species. Floating aquatics (*L. minor* and *A. caroliniana*) were maintained in two of the containers, and also grew on the surface of the water in the containers with rooted species. *L. minor*, *A. caroliniana* and *M. aquaticum* were also maintained in 10-L tanks under fluorescent lighting in the laboratory and fed with Miracle Gro, Standard Methods, or Hoagland's media depending on current experimentation methods. Plants were occasionally obtained from Carolina Biological Supply and some (*e.g.*, *M. aquaticum* and *E. densa*), were collected from the wild. These sources mingled in the simulated lagoon and laboratory maintained cultures.

CHAPTER 4

UPTAKE AND SEQUESTRATION OF ORGANIC CONTAMINANTS BY AQUATIC PLANTS

Contaminant uptake by aquatic plants was experimentally examined with halogenated phenols and *L. minor* and *M. aquaticum*. Results are presented for inactivated-plant and no-plant controls which were used to assess abiotic losses such as photolytic degradation and sorption to plant surfaces. Contaminant uptake by active plant systems was assessed and kinetics of uptake were quantified. Uptake of 15 halogenated phenols containing various halogen substituents were examined to determine effects of halogen type and position on contaminant uptake. Rate of halogenated phenol uptake was compared with predictive relationships for contaminant uptake defined by Briggs and co-workers (1982) and Burken and Schnoor (1998). Physicochemical parameters related to rate of contaminant metabolism by plants were examined to provide additional insight into dynamics of contaminant uptake by aquatic plants.

Photolysis Controls

Photolysis controls were used to quantify effects of light exposure on degradation of contaminants. Batch reactors containing media and 2,4,5-TCP were exposed to light or sequestered in darkness without plant addition to quantify abiotic degradation. Data

presented in Figure 4.1 represent 4.2 mg/L 2,4,5-TCP with no light exposure (i.e., reactor completely covered with aluminum foil allowing no light penetration) and with light exposure typical of plant experimentation (i.e., reactor sidewalls covered and light penetration allowed through media surface). Reactors exposed to light demonstrated a slow, linear decrease in 2,4,5-TCP concentration i.e., 2,4,5-TCP = [4.27 (±0.028)] – [0.0081 (±0.0005)] * time. 2,4,5-TCP concentrations in light exposed reactors were decreased by 18.2% after 90 hr and 9.1% of 2,4,5-TCP was lost over the 48 hr time period typical of experimentation presented herein. Reactors which were not exposed to light exhibited a slow linear increase in 2,4,5-TCP concentration throughout the experiment, where a 4.5% increase in 2,4,5-TCP concentration was observed over 90 hr and a 2.2% increase in 2,4,5-TCP concentration over 48 hr. 2,4,5-TCP losses in light exposed reactors were indicative of photolytic degradation of contaminant. 2,4,5-TCP accumulation in reactors not exposed to light was a result of water loss to the atmosphere and accumulation of contaminants from volatilization of water was considered to be negligible compared to total flux of contaminant in reactors and error associated with TCP measurement. Reactors used to assess uptake of contaminant by plants were assumed to experience smaller losses from photolytic degradation than media control reactors because plant mass reflected and adsorbed a portion the photolytic input provided to media control reactors.

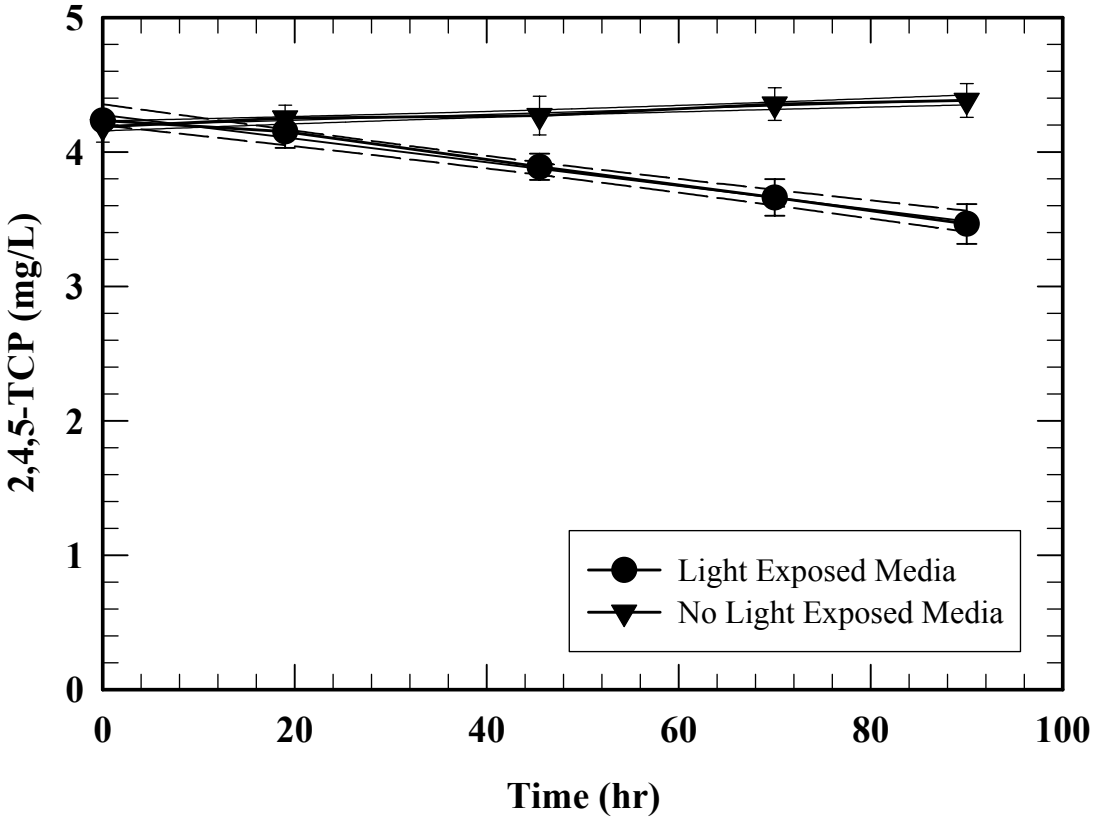


Figure 4.1. Photolysis controls were reactors with media, 2,4,5-TCP and no plant material. Linear regressions of 2,4,5-TCP concentration data are presented with 95% confidence intervals (R^2 values of 0.99) and equations for light exposure and no-light exposure were 2,4,5-TCP = [4.27 (±0.028)] – [0.0081 (±0.0005)]* t and 2,4,5-TCP = [4.19 (±0.014)] – [0.0021 (±0.0002)] * t, respectively.

Inactivated Plant Controls

Inactivated plant controls were used to ensure that contaminant removal was a live-plant mediated process and to quantify sorption to plant surfaces. Inactivated plant controls showed an initial and immediate (i.e., 5 min) decrease in aqueous 2,4,5-TCP concentration upon addition of inactivated *L. minor* to a reactor, followed by 2,4,5-TCP concentration reaching a plateau value (Figure 4.2). Similar sorptive processes occurred upon addition of azide-inactivated *M. aquaticum* to 2,4,5-TCP-reactors, where 2,4,5-TCP was initially removed from reactors after plant addition, followed by 2,4,5-TCP concentrations reaching plateau values (Figure 4.3). Reactors with *M. aquaticum* experienced more abiotic losses than *L. minor* reactors due to an increase in light penetration through media surface. The immediate decrease in concentration found in inactivated systems was comparable to immediate decreases demonstrated in live systems exposed under the same conditions. However, live plant systems demonstrated continued contaminant uptake whereas only the initial contaminant removal was observed in inactivated plant systems (live plant data are presented in subsequent sections). The immediate initial concentration decrease was attributed to contaminant sorption to plants. Contaminant sorption was an extremely rapid process and equilibrium between aqueous and sorbed phases was reached in less than five minutes, as compared to 24 - 48 hr period of contaminant exposure used in experimental systems herein. Contaminant removal from the aqueous phase due to sorption to plants was significant and measurable in both active and inactive plant systems; however contaminant uptake dominated live plant systems and was considered to occur in parallel with sorption.

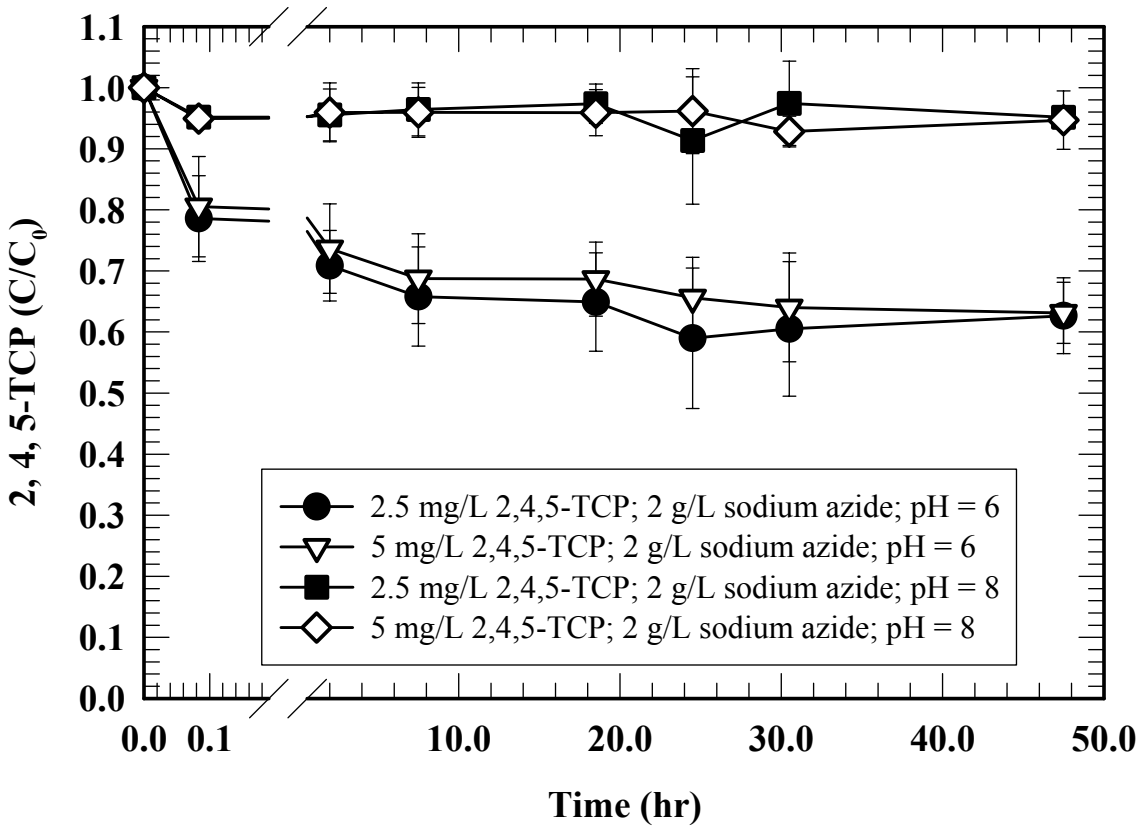


Figure 4.2. 2,4,5-TCP sorption demonstrated at pH values of 6 and 8 and concentrations of 2.5 mg/L and 5 mg/L with inactivated *L. minor*. The split y-axis emphasizes the short time frame over which the majority of sorption occurred.

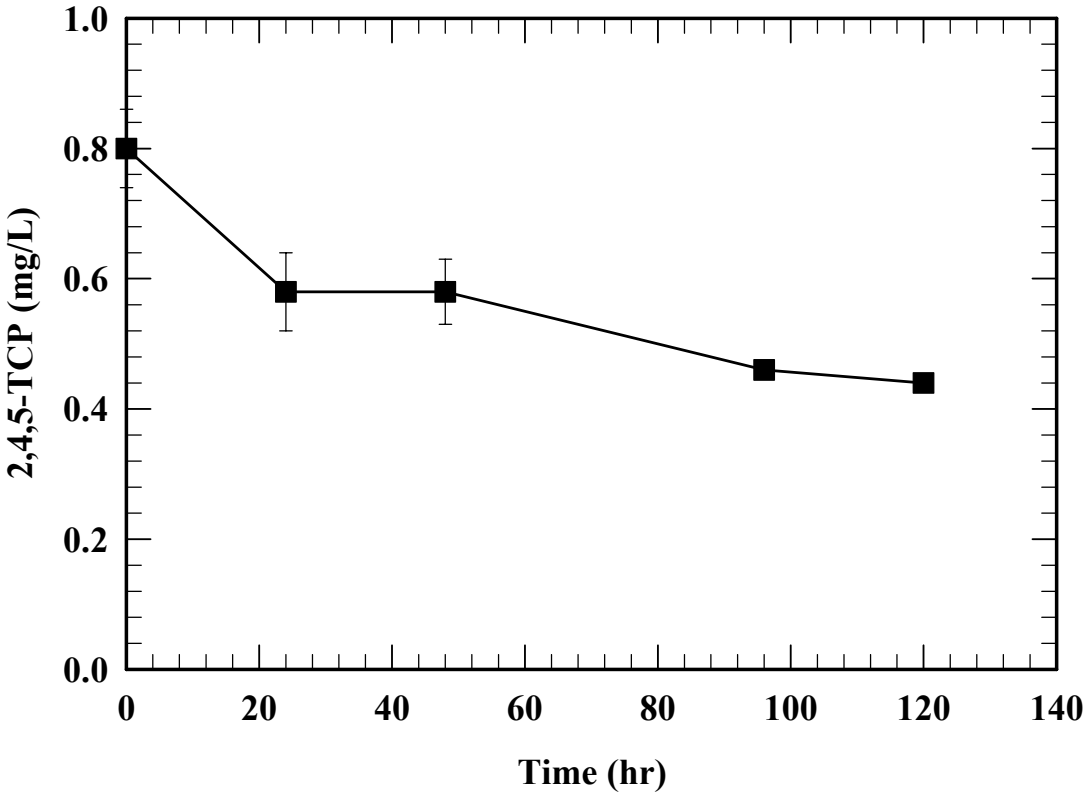


Figure 4.3. Inactivated *M. aquaticum* control demonstrated initial decrease in contaminant concentration followed by slower concentration decrease typical of photolytic degradation.

Data gathered to delineate sorptive behavior of 2,4,5-TCP in plant systems were fitted to a Freundlich isotherm, as compared to linear and Langmuir isotherms. In the Freundlich isotherm, $q_e = K_f S^n$, q_e is mass of contaminant sorbed (mg/g), S is aqueous phase concentration (mg/L), K_f and n are parameters indicative of sorption capacity and energies associated with sorption, respectively (Weber, 2001). The Freundlich isotherm for 2,4,5-TCP plant systems (Figure 4.4) was developed between concentration of protonated 2,4,5-TCP (mg/L) and mass of sorbed 2,4,5-TCP (mg/g). Values used to develop the isotherm were from measurements in live and inactivated systems taken five minutes after addition of plants to reactors, and no variation was observed between active and inactive systems. Four media pH values were used to examine sorption to plants (Figure 4.5). Low media pH values comprised the majority of the curve, with data for higher pH values (8 and 9) at concentrations of TCP in the protonated form of less than 1 mg/L. Data for higher pH values were for lower concentrations of TCP in protonated form because the pK_a of TCP is 7.0, and therefore only 90.9% of TCP at pH 8 is in the protonated form (99.0% at pH 9) resulting in low concentrations of TCP in protonated form when TCP concentrations that are not-toxic to plants were examined. Parameters found for 2,4,5-TCP in plant systems were $K_f = 0.021 \pm 0.001$and $n = 0.574 \pm 0.054$. The n value was in the range of parameters previously observed (Speth and Miltner, 1998; Dobbs and Cohen, 1980; Faust and Aly, 1987) for sorption of substituted phenolic compounds to activated carbon (2-chlorophenol: n = 0.41; 2,4,6-trichlorophenol: n = 0.4; n = 0.34; 2-nitrophenol: n = 0.34; 2,4-dimethylphenol: n = 0.44). The similarity with activated carbon was used not because activated carbon is plant-like, but because the chlorophenols examined with activated carbon were representative of those examined for

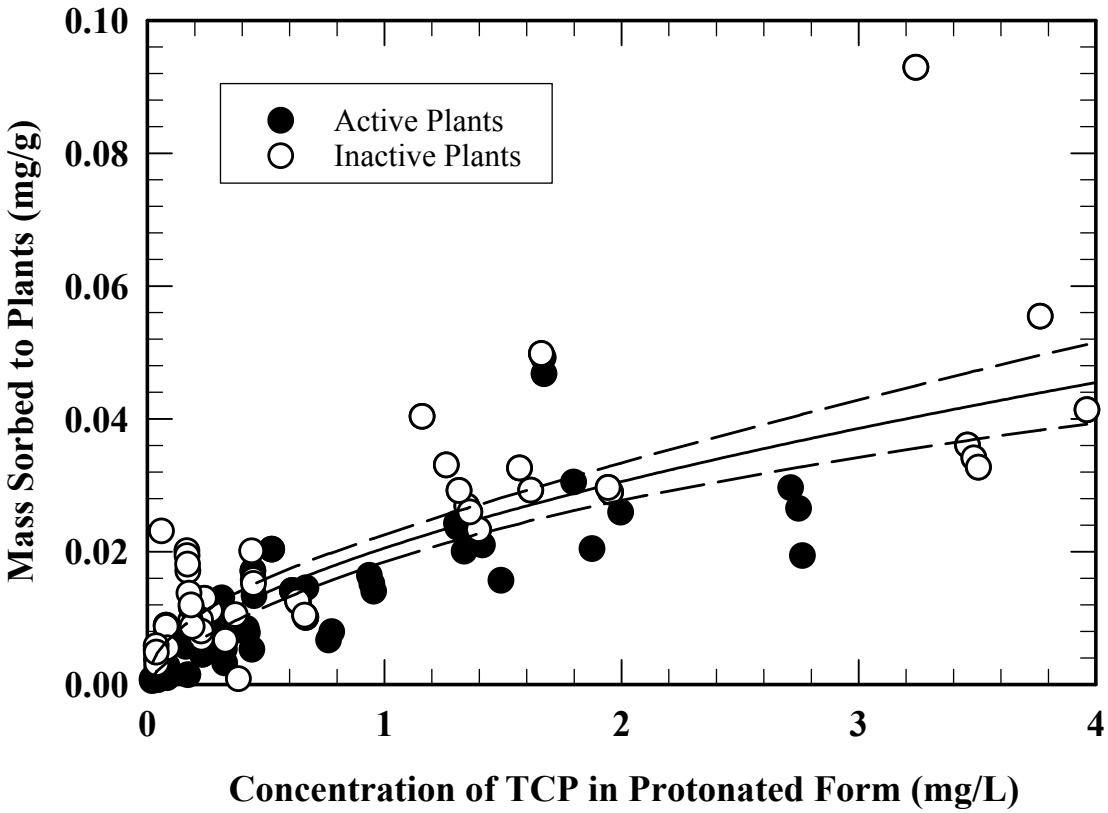


Figure 4.4. Freundlich isotherm for protonated 2,4,5-TCP sorption to active and inactive plants. The non-linear regression (K_f = 0.021 (±0.001), n =0.574 (±0.054); R^2 = 0.59) is shown with 95% confidence intervals. The t-statistic produced two-sided p-values < 0.0001, indicating that both parameters were valid. (Note: media pH values for sorption data are presented in Figure 4.5).

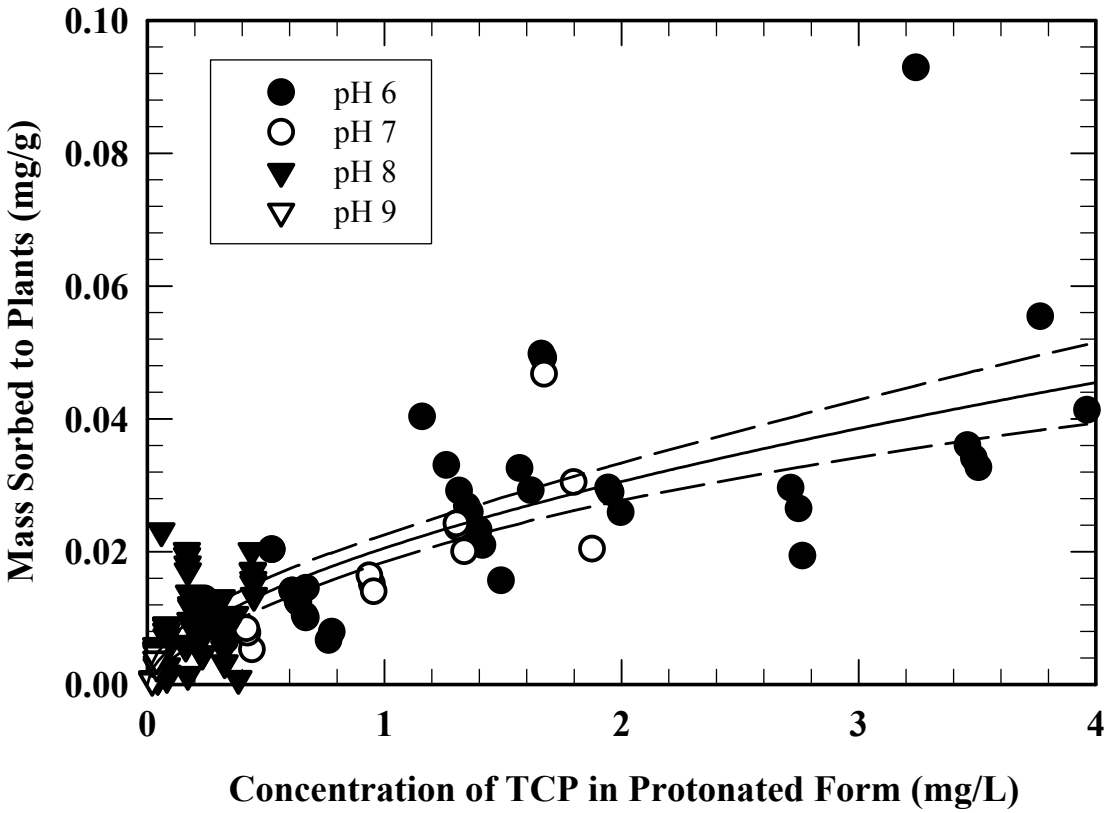


Figure 4.5. Freundlich isotherm for protonated 2,4,5-TCP sorption to active and inactive plants exposed with symbols delineating media pH values. (Note: active and inactive plants for sorption data are delineated in Figure 4.4).

partitioning onto plant surfaces in this study. These studies were used to confirm that continued uptake did not occur in inactivated plant systems and that sorption to plant surfaces played an important role in contaminant removal.

The Freundlich isotherm developed for use throughout the remainder of the thesis was developed with a portion of the data presented in Figure 4.4 and 4.5. The dataset in Figures 4.4 and 4.5 contains two types of data: the data used to parameterize the model presented subsequently and independent data which were used to verify model predictions. Therefore, the sorption isotherm used in the model contained only data used to parameterize the model, not those gathered for model verification. The isotherm used in the model is presented in Figure 4.6 (active and inactive data delineated) and Figure 4.7 (media pH values delineated). Model parameters for sorption to plants were K_f = 0.023 (±0.001) and n = 0.53 (±0.075). Isotherm parameters used in the model were not statistically different from isotherm parameters developed for all data, previously presented in Figures 4.4 and 4.5, which were K_f = 0.021 (±0.001) and n = 0.574 (±0.054).

Active Plant Uptake of Organic Contaminants

A suite of aquatic plant species were screened for ability to uptake organic contaminants and ability to withstand toxic effects of contaminants at low levels typical of natural environment systems. *L. minor, M. aquaticum, E. nuttali, A. carolina* and *C. demersum* were screened for ability to withstand and uptake concentrations relevant to the natural environment. Efforts were focused on uptake of substituted phenols by *L. minor* and

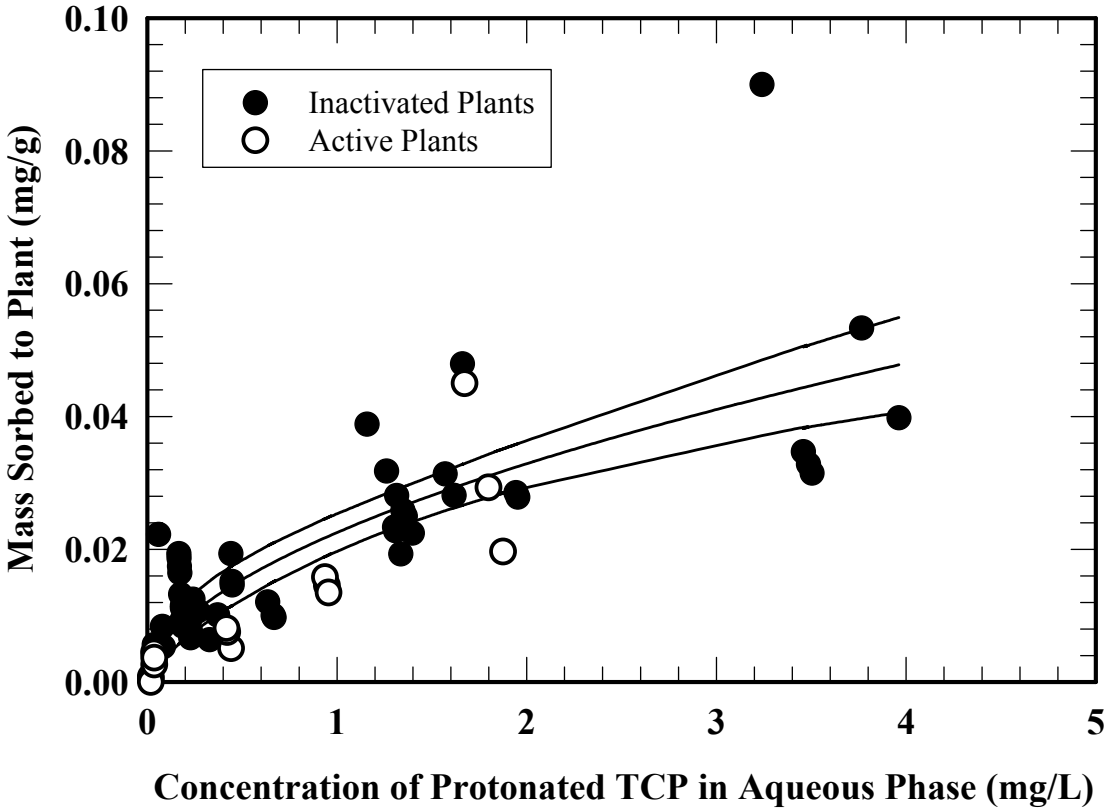


Figure 4.6. Freundlich isotherm for protonated 2,4,5-TCP sorption to active and inactivated plants. The non-linear regression (K_f = 0.023 (±0.001), n =0.53 (±0.075); R^2 = 0.65) is shown with 95% confidence intervals. The t-statistic produced two-sided p-values < 0.0001, indicating that both parameters were valid.

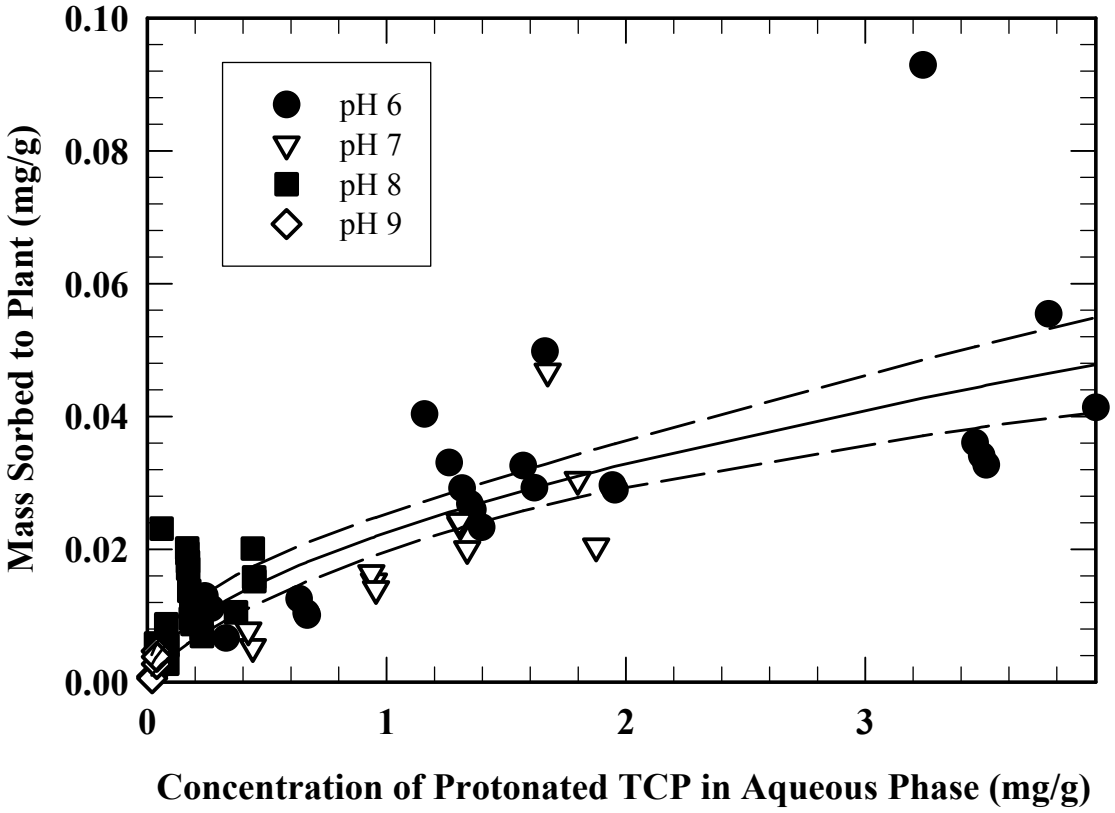


Figure 4.7. Freundlich isotherm for protonated 2,4,5-TCP sorption to active and inactivated plants with media pH values delineated. The non-linear regression (K_f = 0.023 (±0.001), n =0.53 (±0.075); R^2 = 0.65) is shown with 95% confidence intervals. The t-statistic produced two-sided p-values < 0.0001, indicating that both parameters were valid.

M. aquaticum because of their relatively hardy nature and ability to grow rapidly. *L. minor* was a particular focus because of its extensive use in toxicity assay.

Contaminant Uptake by *L. minor*.

Batch reactors were used to assess the ability of *L. minor* to uptake chlorophenols. Uptake of chlorophenols by *L. minor* was a rapid, first-order process and example data illustrating uptake of 2,4,5-TCP by *L. minor* are provided in Figure 4.8. Plant exposure to three initial concentrations (C_0 = 0.46, 0.89 and 2.59 mg/L) of 2,4,5-TCP are shown (Figure 4.8) for systems with a media pH value of 6 and 15.7 g FW/L of plant mass. 2,4,5-TCP was rapidly removed from aqueous phase by plants for all three concentrations shown in Figure 4.8 and 2,4,5-TCP was totally depleted from aqueous phase by 48 hr in low concentration systems (i.e., C_0 = 0.46 and 0.89 mg/L). A rapid and immediate decrease in 2,4,5-TCP concentration was observed in all systems, where a 5 – 20% decrease in contaminant concentration was observed between the initial data point (t = 0) and the first data point (t = 5 min). The kinetics of the initial rapid removal are emphasized in Figure 4.9 which displays data from Figure 4.8 with a break the time-axis that highlights time points less than 10 min. These data demonstrated the immediate and rapid nature of the initial contaminant removal, a concept consistent with sorption to plant surfaces in inactivated systems, as was presented previously. Contaminant sorption was recognized to be significant and measurable in active and inactive plant systems; however contaminant uptake dominated live plant systems and was considered to occur in parallel with sorption, whereas sorption dominated inactive plant systems. In active plant systems, contaminant uptake proceeded at a first-order rate subsequent to rapid

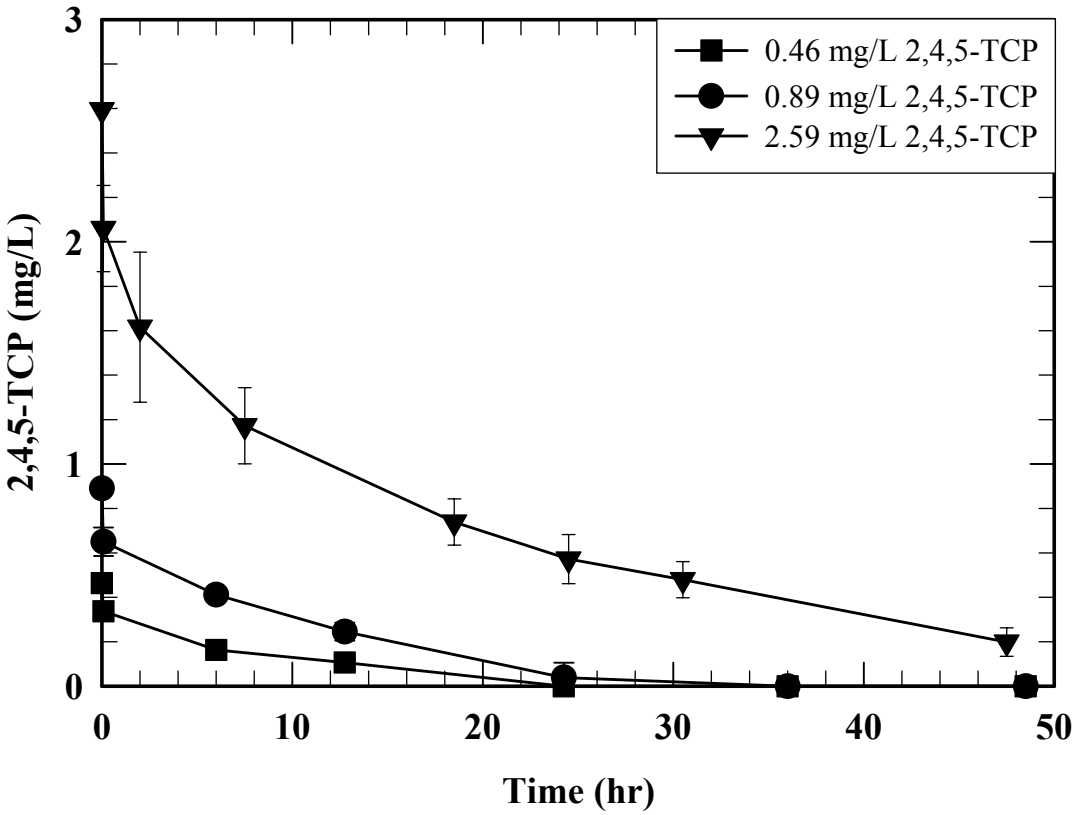


Figure 4.8. Rapid, first-order uptake of three initial concentrations of 2,4,5-TCP by *L. minor* with media at a pH value of 6.

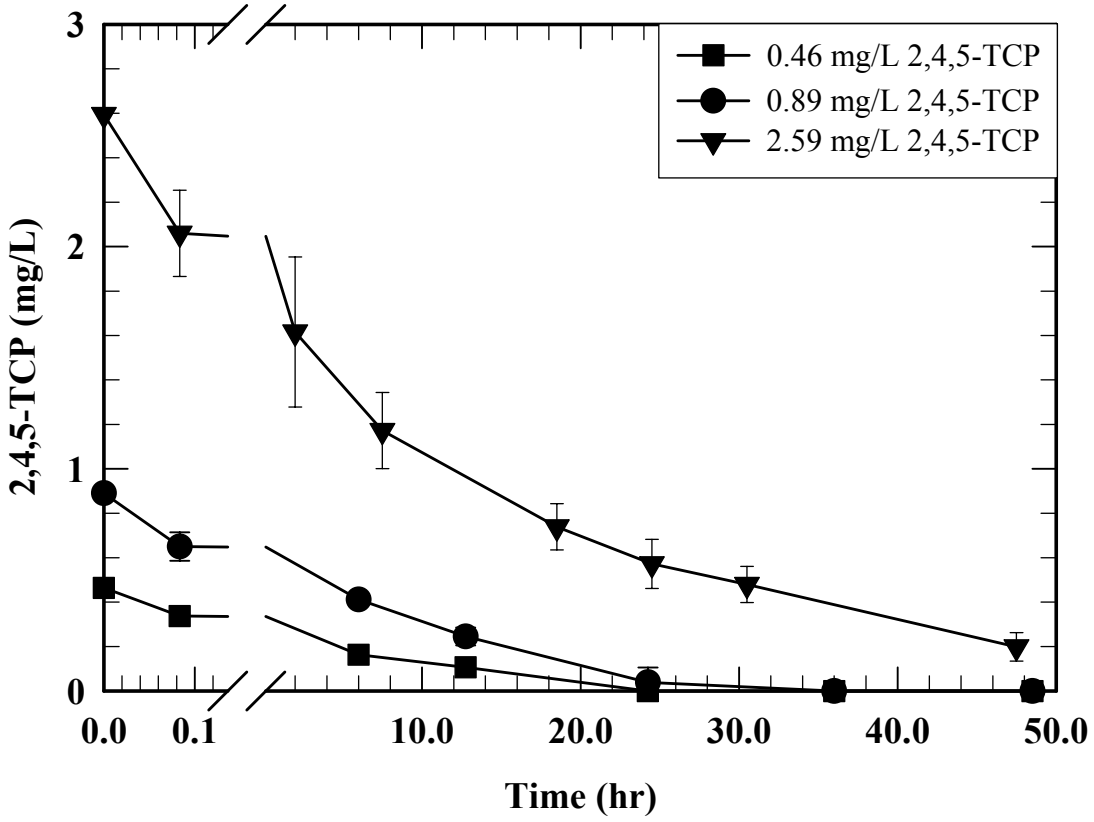


Figure 4.9. Rapid and immediate (i.e., t<5 min) decrease in 2,4,5-TCP concentration is highlighted with data presented in Figure 4.5 by a break in the x axis to clearly show kinetics at t<10 min.

initial sorption. Therefore, data for plant systems provided evidence for a two-step mechanism where plant-contaminant interactions occur in two steps, a fast step represented by equilibrium sorption, and a slow step represented by first-order uptake rates. It is reasonable to conjecture that contaminants interact with plants in two regions and a two-site model was used for description of plant-contaminant interactions. The two-step mechanism is consistent with adsorption in abiotic systems. In one example, researchers examined sorption of vanadyl and chromium to aluminum oxide and observed rapid initial adsorption followed by a slow adsorption step that was dependant on pH (Wehrli et al., 1990).

First-order, non-linear regression of contaminant concentration with time was used to model kinetic rate of contaminant uptake by plants. The initial data point (i.e., t=0) was excluded from first-order regression analysis due to the rapid initial concentration decrease observed between the initial data point (t = 0) and the first data point (t = 5 min). This treatment of the data effectively removed initial sorption-effects from the kinetic assessment. Example first-order non-linear regression curves are shown in Figure 4.10 for each data set presented previously in Figures 4.8 and 4.9. The first-order rate coefficient, k, was used to quantify contaminant uptake rate and k values are shown for example data with corresponding non-linear regression curves in Figure 4.10. Further examination of factors which influence k values is presented in subsequent sections. An initial concentration was also fit to contaminant uptake data ($C_{0,\ fitted}$) when k values were generated. Because the initial data points (t = 0) were excluded from analyses, the $C_{0,\ fitted}$ values very closely matched the concentration measured at t = 5 min. Values for $C_{0,\ fitted}$

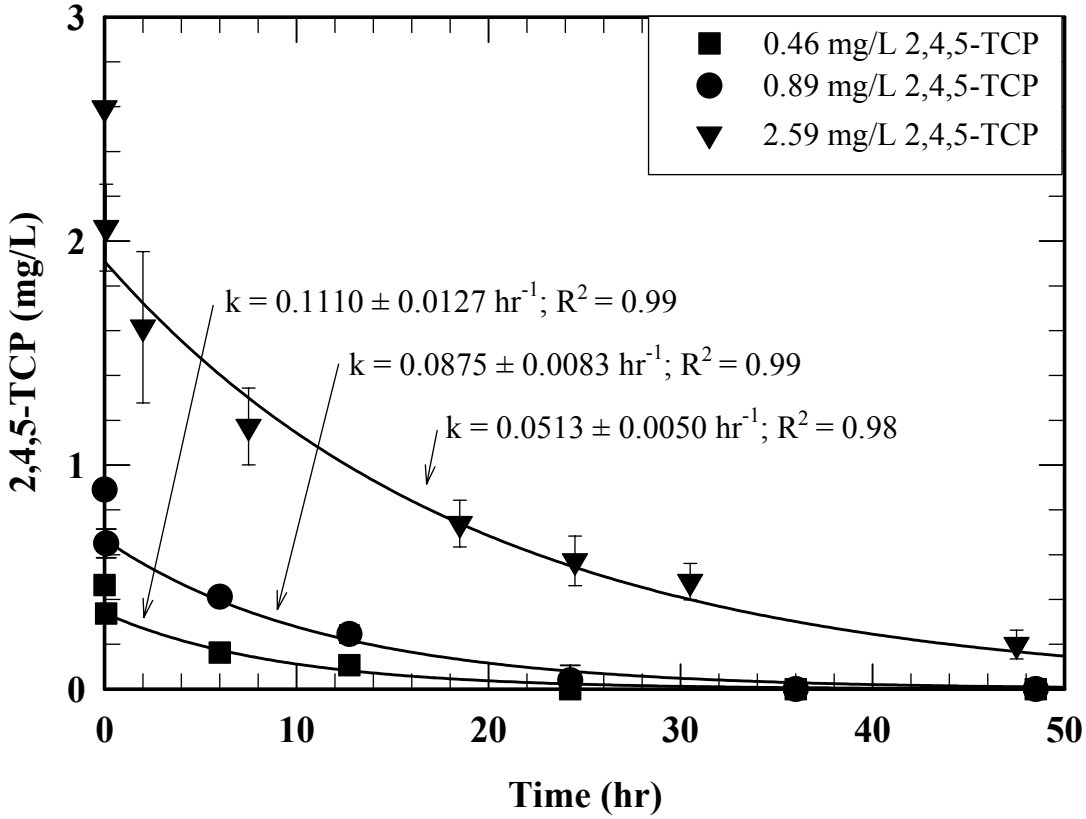


Figure 4.10. Example first-order non-linear regression curves are provided for 2,4,5,-TCP removal at three concentrations. The first-order rate coefficient, k, was used to quantify uptake rate and values are provided for regressions shown.

for example data previously present in Figures 4.8 - 4.10 for are presented in Table 4.1. It is clear from examination of these data that $C_{0, fitted}$ values closely correspond with concentrations measured at t = 5min and do not match concentrations measured before addition of plants to reactors. Therefore, curve fitting results for C_0, fitted and k were consistent with first-order loss of contaminant from aqueous phase and exclusion of initial (t = 0) data points from curve fitting.

Solvent Enhanced Contaminant Uptake. Addition of small volumes of acetone to media was observed to enhance contaminant uptake rate. Elevated k values were observed for reactors with solvent addition compared to reactors with identical exposure conditions without solvent addition. A comparison of contaminant uptake in an acetone amended (media = 1% acetone) system with a no-solvent amended system is presented in Figure 4.11. Non-linear regression analysis provided k values of 0.0137(±0.0005) hr^{-1} and 0.0282 (±0.0012) hr^{-1} for acetone amended and no-acetone-amended systems, respectively. These results demonstrated that contaminant uptake was significantly faster in acetone amended systems than in no-solvent amended systems, where results indicated the complicated nature of multiple contaminant systems. In addition, uncertainty observed in acetone amended systems was significantly greater than that of no-acetone-amended systems. However, no further investigation into solvent enhanced uptake was made because of negative plant health implications associated with solvent addition.

Photoperiod Effects on Contaminant Uptake. Effects of cycled versus continuous light on contaminant uptake were examined to investigate potential links between

Table 4.1. Values of $C_{0,\ fitted}$ closely correspond to the concentration measured at t = 5 min and do not agree with initial concentrations.

Initial Concentration (mg/L)	Concentration t = 5 min (mg/L)	$C_{0,\ fitted}$	R^2
0.46	0.34	0.34 (± 0.02)	0.98
0.89	0.65	0.68 (± 0.03)	0.99
2.59	2.06	1.91 (± 0.08)	0.99

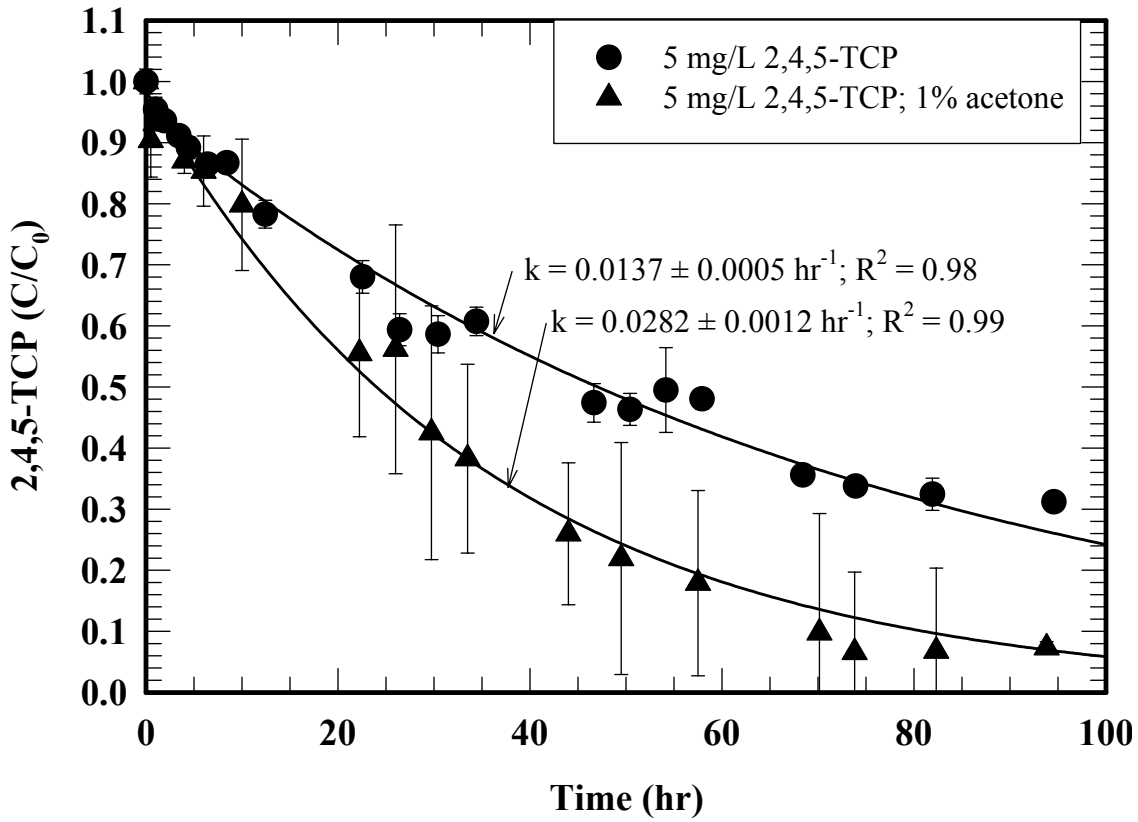


Figure 4.11. 2,4,5-TCP uptake by *L. minor* at 5 mg/L with additions of small volume of acetone (1% v/v).

photosynthetic or respiratory activity and contaminant uptake and to investigate the potential for light mediated contaminant processing reactions. Reactors which contained 5 mg/L 2,4,5-TCP and 15.7 g FW/L *L. minor* were exposed to continuous light or light cycled at 16 hr light and 8 hr dark. No change in contaminant uptake rate was observed between plants exposed to continuous light and plants exposed to cycled light as was demonstrated by data presented in Figure 4.12. The k values for continuous and cycled light exposure were 0.0116 ± 0.0004 hr^{-1} and 0.0137 ± 0.0005 hr^{-1}, respectively. Although k values were statistically distinct, data for continuous light exposure continuously overlapped with those of cycled light exposure, therefore, no significant difference was considered to occur in uptake rate when photoperiod was altered.

Contaminant Uptake by *M. aquaticum*.

The ability of *M. aquaticum* to uptake chlorophenols was assessed using batch reactors. *M. aquaticum* was observed to rapidly uptake chlorinated phenols with first-order kinetics that paralleled the kinetics of *L. minor* uptake of chlorinated phenols. Example data demonstrating 2,4,5-TCP removal at concentrations of 10 and 50 mg/L from the aqueous phase by *M. aquaticum* are provided in Figure 4.13. Contaminant uptake was observed in 10 mg/L reactors (>30% removal by 140 hr). In contrast, no contaminant uptake was observed in 50 mg/L reactors (<5% removal by 140 hr) where plants were assumed to be inactivated by 2,4,5-TCP. Additional data for uptake of 2,4,5-TCP by *M. aquaticum* are provided in Figure 4.14, where initial contaminant concentration was 1 mg/L. When *M. aquaticum* was exposed at this low concentration, contaminant uptake proceeded throughout the course of the experiment ($k = 0.051 \pm 0.004$) and all

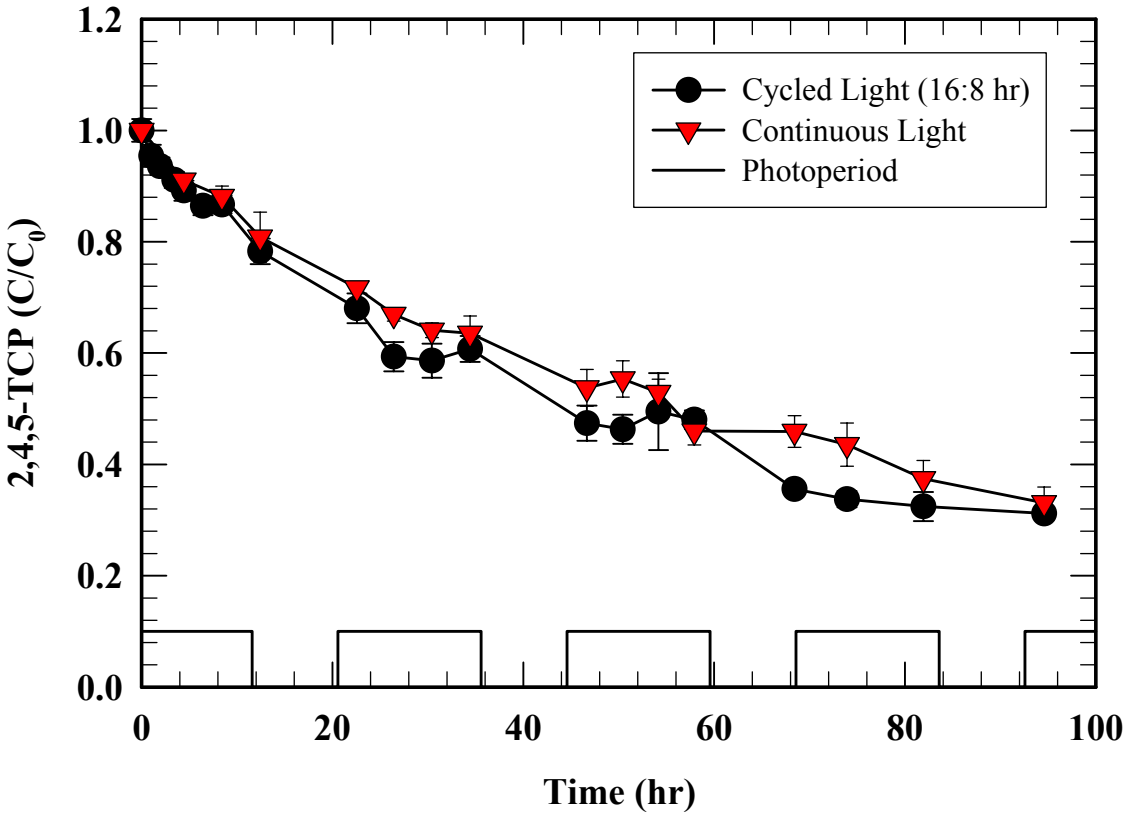


Figure 4.12. Examination of photoperiod effects on contaminant uptake for *L. minor* exposed to 5 mg/L 2,4,5-TCP. Bars at the base of the figure represent light and gaps represent dark in cycled light system.

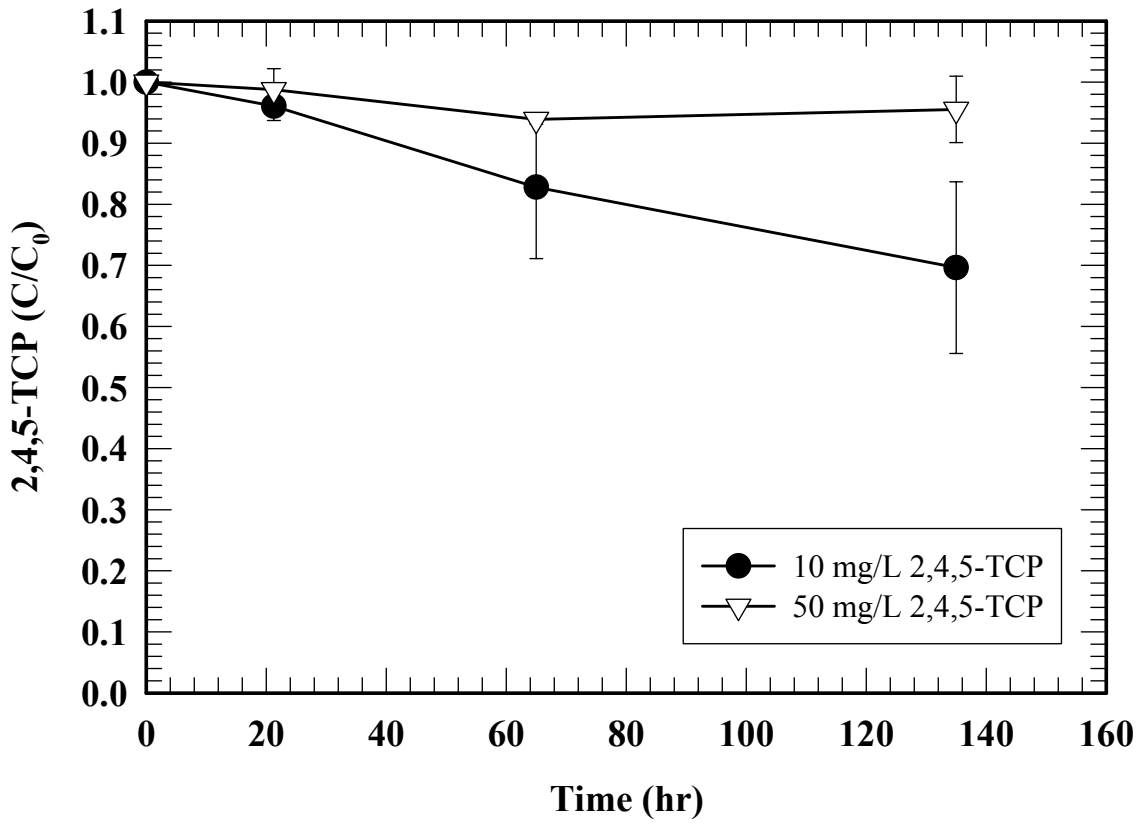


Figure 4.13. 2,4,5-TCP uptake by *M. aquaticum* at 10 mg/L and 50 mg/L.

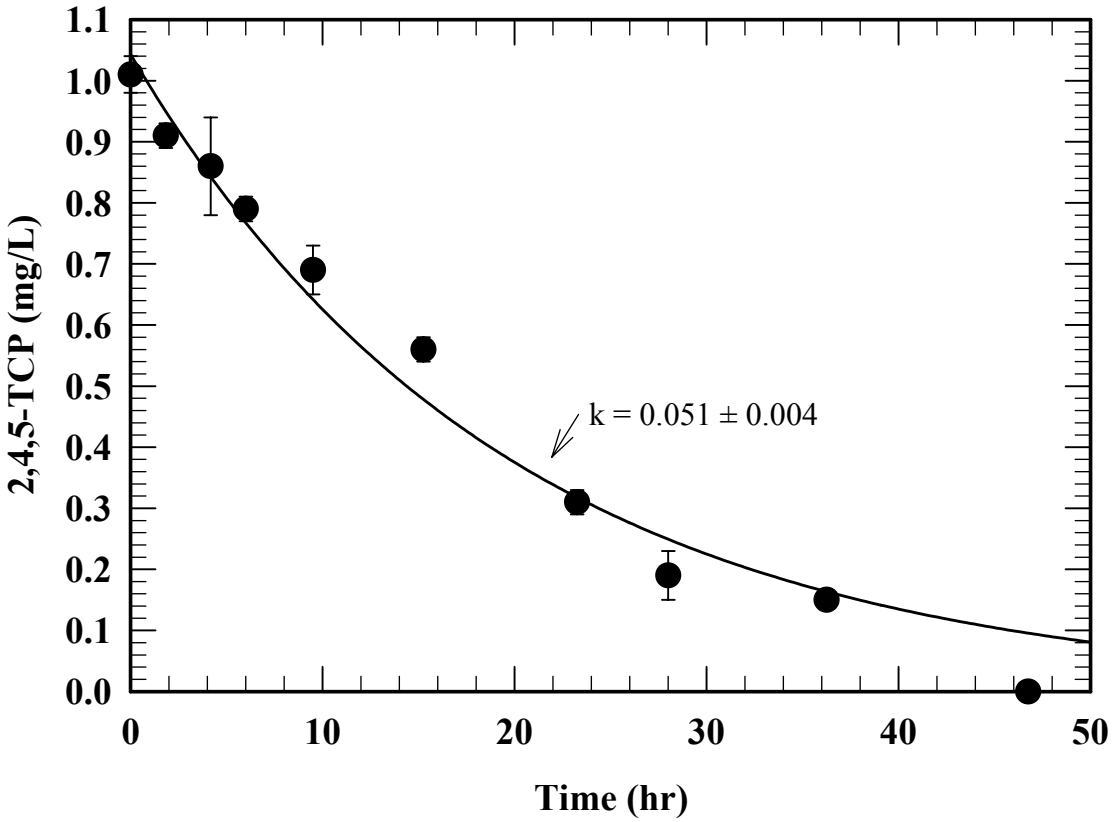


Figure 4.14. Uptake of 2,4,5-TCP by *M. aquaticum* exposed to 1 mg/L. First-order nonlinear regression and rate constant provided. Complete contaminant removal was observed by 48 hr.

contaminant was depleted from aqueous phase by 48 hr. The ability of multiple excised *M. aquaticum* shoots to uptake contaminant was compared with that of a whole *M. aquaticum* plant. Data presented in Figure 4.15 compare uptake by five shoots excised several cm below water level with uptake by a whole plant, where the five shoots were equal in mass to the whole plant. Mass removed by 24 hr was greater in the multiple shoot system, however the rate of uptake was not enhanced by increasing the number of shoots in the reactor relative to the total mass of plant exposed. k values were used to quantify this results, where $k = 0.0306 \pm 0.0039\ hr^{-1}$ for whole plant systems and $0.0406 \pm 0.0048\ hr^{-1}$ in multiple shoot systems. In addition, both the multiple shoot system and the whole plant system removed significantly more contaminant that was removed in the sodium azide control.

Effect of Chemical Structure and Physicochemical Parameters on Rate of Halogenated Phenols Uptake by Aquatic Plants

Uptake of halogenated phenols by *L. minor* and *M. aquaticum* has been demonstrated. In an effort to more fully understand factors which control uptake of halogenated phenols by aquatic plants, phenols containing various halogen substituents were examined with *L. minor* to determine effects of halogen type and position on contaminant uptake. Predictive relationships for contaminant uptake developed in terrestrial-plant systems and based on partitioning processes were examined for use in aquatic plant systems. Finally, physicochemical parameters related to rate of contaminant metabolism by plants were examined to provide additional insight into dynamics of contaminant uptake by aquatic plants.

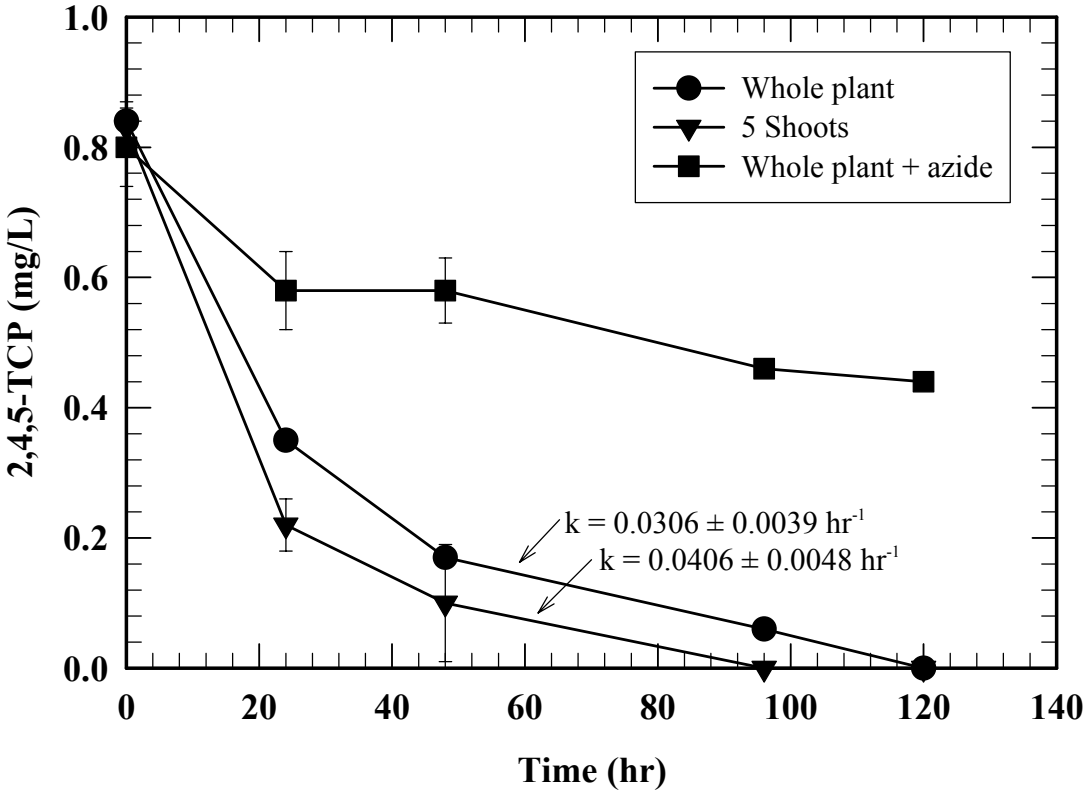


Figure 4.15. Uptake rate was compared between a whole *M. aquaticum* plant (50 g FW/L) and 5 shoots (total weight = 50 g FW/L). Greater initial removal was observed in the multiple shoot system than in whole plant system, however uptake rates after initial data points were not statistically distinct.

Halogenated Phenols Examined

Batch experiments (1 g plant, 100 mL media) were used to examine effects of chemical structure and related physical and chemical parameters on contaminant uptake rate. *L. minor* was exposed to fluorinated, chlorinated and brominated phenols with varying substituent position and number. Three trichlorinated phenols were studied, 2,4,5-TCP, 2,4,6-trichlorophenol (2,4,6-TCP) and 2,3,5-trichlorophenol (2,3,5-TCP) along with tri-halogenated analogs 2,3,5-trifluorophenol (2,3,5-TFP), 2-chloro-3,5-difluorophenol (2-Cl-3,5-DFP) and 2,4,6-tribromophenol (2,4,6-TBP). Two dichlorophenols were examined, 2,4-DCP and 2,6-dichlorophenol (2,6-DCP) along with four analogs, 2-chloro-4-fluorophenol (2-Cl-4-FP), 4-fluoro-2-chlorophenol (4-Cl-2-FP), 2,6-difluorophenol (2,6-DFP), 3,5-difluorophenol (3,5-DFP). 4-chlorophenol (4-CP) and 4-fluorophenol (4-FP) were studied along with 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP). A list of halogenated phenols examined is provided subsequently in Table 4.2.

Kinetic studies examining rate of halogenated phenol uptake utilized 1 g of *L. minor* exposed to various halogenated phenols in 100 mL of media. Media was titrated to pH 5 so that media pH was at least a full pH unit lower that the lowest pK_a examined (pK_a values ranged from 6.04 – 9.54). Thus, fraction protonated (*f*) was greater than 0.90 in all systems. Initial concentrations of halogenated phenols ranged nominally from 7 – 12 μmol/L, with initial concentrations of 2.3 and 25.7 μmol/L for 3,5-difluorophenol and 4-chlorophenol, respectively. Initial concentrations were targeted to be below toxicity thresholds observed for 2,4,5-TCP at pH 6 as discussed in subsequent chapters. All halogenated phenol concentrations were significantly below reported solubility limits in

water (Howard and Meylan, 1997) and singular halogenated phenols were used at all times (i.e., chemical mixtures were not within the scope of this study).

All plants used for examination of effects of chemical and physical properties on halogenated phenol uptake were taken from a singular, uniform plant stock culture at the same point in time. Therefore plant metabolic processes were considered to be uniform in all experiments used to examine effects of chemical and physical properties on halogenated phenol uptake by aquatic plants. Oxygen production rate measurements were used to verify that all systems in this study were not inhibited relative to controls, as described in subsequent chapters. 2,3,4,6-TeCP was used as a positive control for inhibition measurements and results verified that toxic effects were measurable in this system. Because toxic effects were observed in the 2,3,4,6-TeCP system, data gathered for 2,3,4,6-TeCP were not used in kinetic analysis.

Calculation of Molecular Physicochemical Properties

Molecular physiochemical properties were determined for comparison with contaminant uptake rate. Literature K_{ow} values were used when available and unreported K_{ow} values were calculated with the KOWWIN program supplied by EPA (Environmental Protection Agency, 2002). Acidity (i.e., pK_a) was determined by SPARC using the online calculator. Molecular connectivity was determined using the WinTox program (Jorgensen *et al.,* 1998).

Inactivated Controls

Inactivated control experiments were performed for 2,4,5-TCP and were presented previously in Figure 4.2. Results showed that an initial and immediate decrease in aqueous concentration upon addition of inactivated plants to a reactor, followed by 2,4,5-TCP concentrations reaching a plateau value. The immediate decrease in concentration found in inactivated systems was comparable to the immediate decrease seen in live systems exposed to 2,4,5-TCP under the same conditions; however live plant systems demonstrated continued 2,4,5-TCP uptake whereas only the initial contaminant removal was observed in inactivated plant systems. Inactivated control data demonstrated that contaminant uptake only occurred in live-plant systems. It was assumed that parallel processes occurred with all halogenated phenols, therefore continued decrease in contaminant concentrations was attributed to uptake by plants.

Plant Uptake of Halogenated Phenols

Variations in halogen-type, substituent number and substituent position affected rate of contaminant uptake by *L. minor*. Data and first-order, non-linear regression projections for systems that exhibited rapid uptake are shown in Figures 4.16 – 4.19. Uptake of halogenated phenol from the aqueous phase was a rapid, first-order process in most halogenated phenol systems examined. First-order, non-linear regression was performed for each exposure as described previously and k values quantifying rate of contaminant uptake were generated. Average k values for each contaminant examined are provided in Table 4.2 with the standard deviation of the k values determined for triplicate reactors. All halogenated phenols tested produced k values that were statistically significant (i.e.,

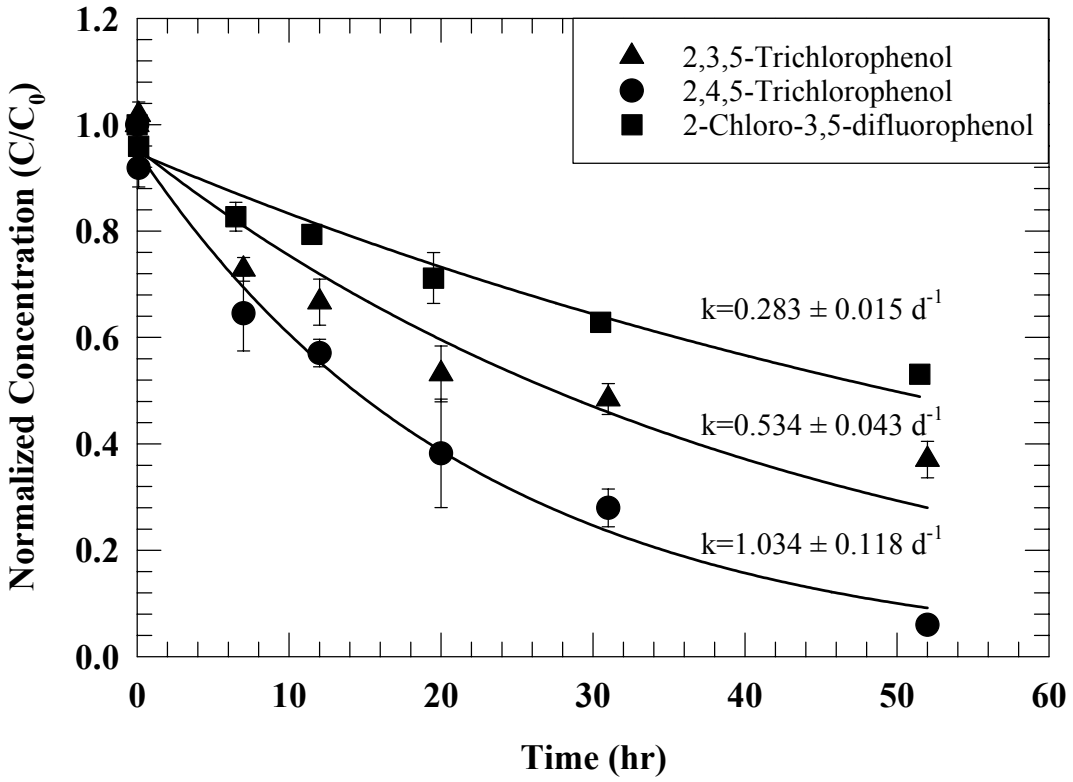


Figure 4.16. Contaminant uptake rates provided for tri-halogenated phenol systems where rapid uptake was observed. Data shown represent plants exposed to similar initial halogenated phenol concentrations at pH 5 that possessed identical plant activity. The first-order rate coefficient, k, found through non-linear regression is indicated for each contaminant.

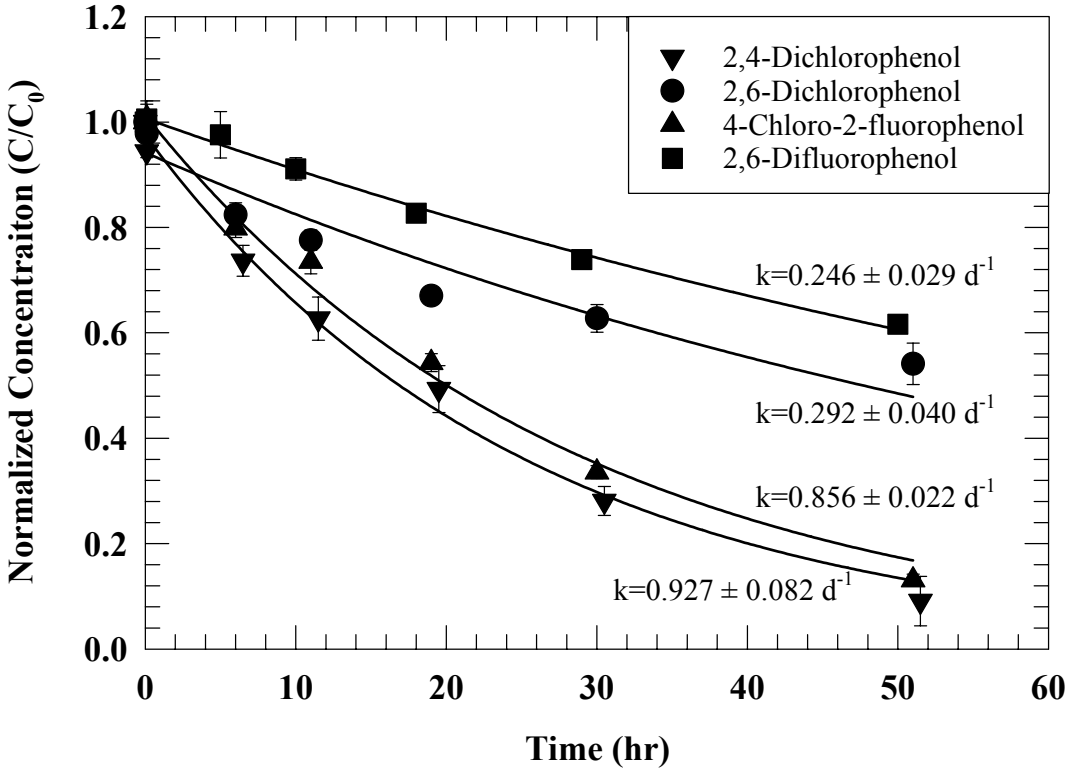


Figure 4.17. Contaminant uptake rates provided for di-halogenated phenol systems where rapid uptake was observed. Data shown represent plants exposed to similar initial halogenated phenol concentrations at pH 5 that possessed identical plant activity. The first-order rate coefficient, k, found through non-linear regression is indicated for each contaminant.

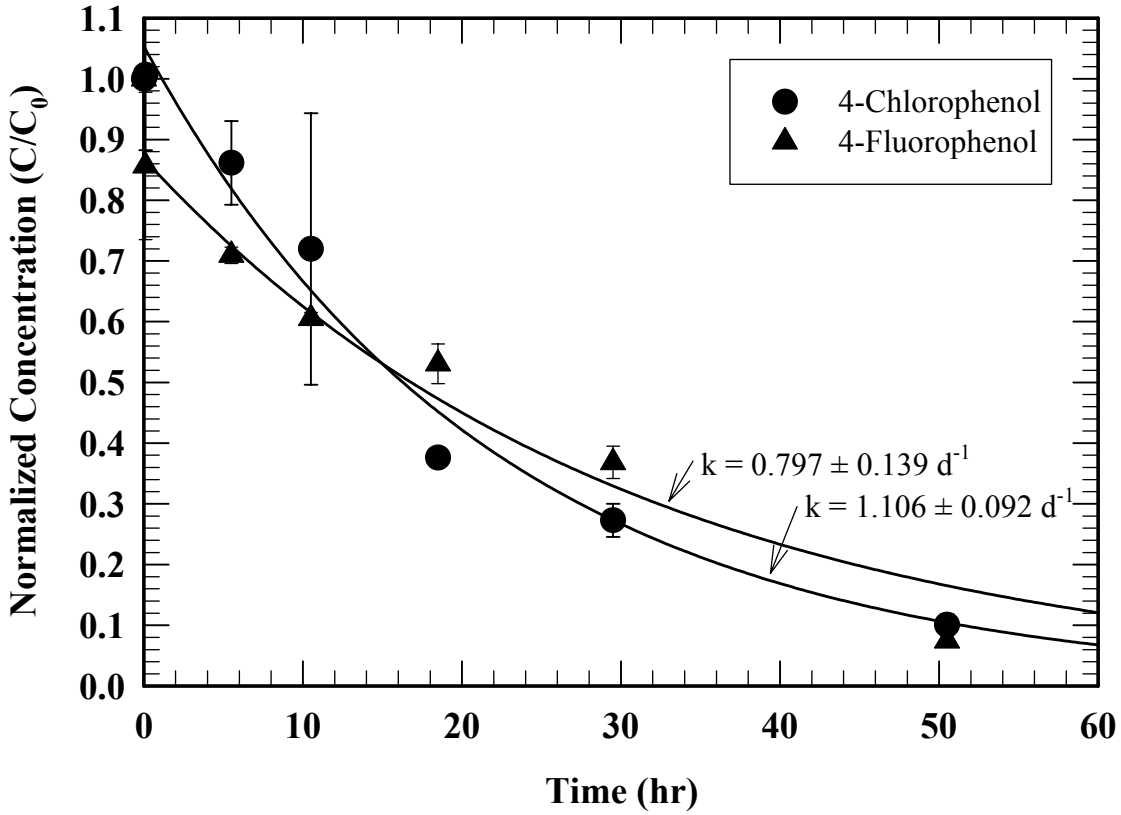


Figure 4.18. Contaminant uptake rates provided for mono-halogenated phenol systems where rapid uptake was observed. Data shown represent plants exposed to similar initial halogenated phenol concentrations at pH 5 that possessed identical plant activity. The first-order rate coefficient, k, found through non-linear regression is indicated for each contaminant.

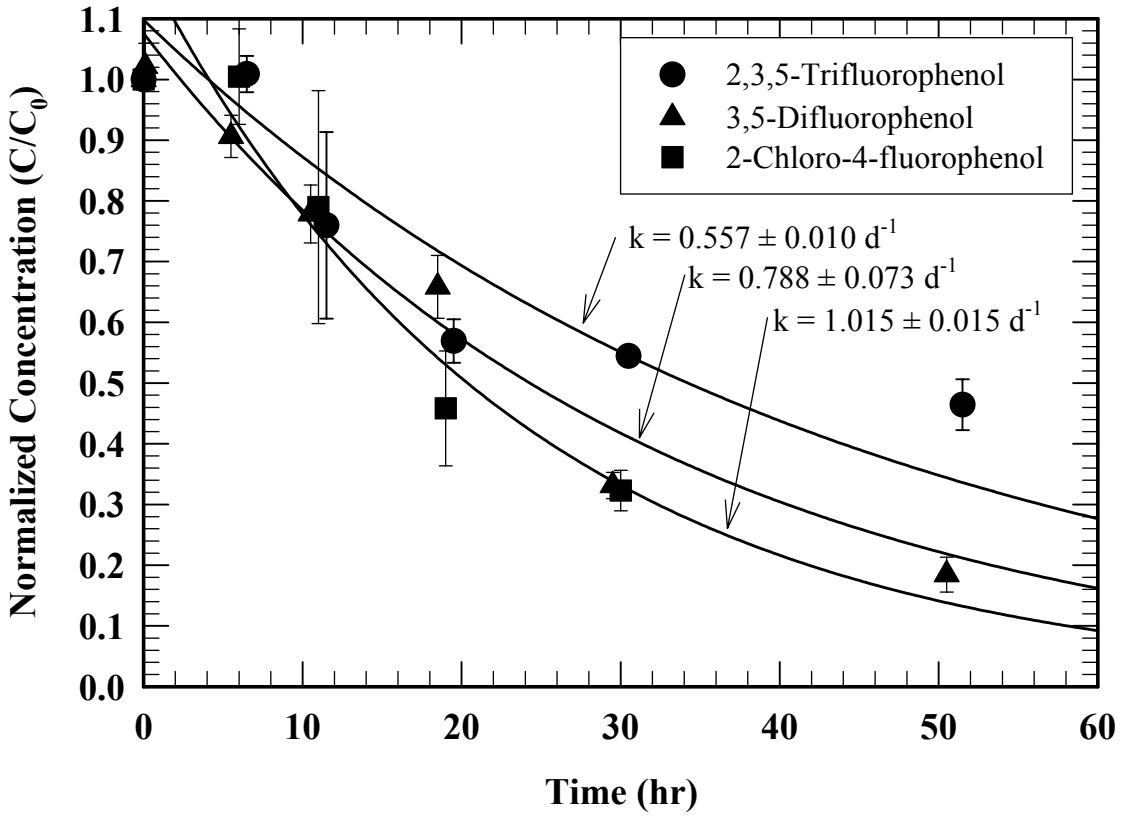


Figure 4.19. Contaminant uptake rates provided for example reactor systems where rapid uptake was observed. Data shown represent plants exposed to similar initial halogenated phenol concentrations at pH 5 that possessed identical plant activity. The first-order rate coefficient, k, found through non-linear regression is indicated for each contaminant.

Table 4.2. Halogenated phenol experimental data.

Compound Name	CAS-RN	MW (g/mol)	C_0 (μmol/L)	Average k (d^{-1})	pK_a	Log K_{ow}
2,3,5-Trichlorophenol (2,3,5-TCP)	933-78-8	197.5	7.9	0.534±0.043	6.62	3.84[b]
2,4,5-Trichlorophenol (2,4,5-TCP)	95-95-4	197.5	8.0	1.034±0.118	6.86	3.72[b]
2,4,6-Trichlorophenol (2,4,6-TCP)	88-06-2	197.5	10.5	0[a]	5.96	3.69[b]
2,3,5-trifluorophenol (2,3,5-TFP)	2268-15-7	148.1	12.0	0.557±0.010	7.11	2.11[c]
2-chloro-3,5-difluorophenol (2-Cl-3,5-DFP)	206986-81-4	181.0	10.5	0.283±0.015	6.88	2.56[c]
2,4-dichlorophenol (2,4-DCP)	120-83-2	163.0	7.3	0.927±0.082	7.68	3.06[b]
2,6-dichlorophenol (2,6-DCP)	87-65-0	163.0	7.3	0.292±0.040	6.53	2.75[b]
2-chloro-4-fluorophenol (2-Cl-4-FP)	1996-41-4	146.6	9.6	1.015±0.015	7.80	2.36[c]
4-chloro-2-fluorophenol (4-Cl-2-FP)	348-62-9	146.6	9.3	0.856±0.0217	7.92	2.36[c]
3,5-difluorophenol (3,5-DFP)	2713-34-0	130.1	12.3	0.788±0.073	8.62	1.91[c]
2,6-difluorophenol (2,6-DFP)	28177-48-2	130.1	2.3	0.246±0.029	6.98	1.96[c]
4-chlorophenol (4-CP)	106-48-9	128.6	25.7	0.797±0.139	9.43	2.39[b]
4-fluorophenol (4-FP)	371-41-5	112.1	14.7	1.106±0.092	9.54	1.77[b]
2,4,6-tribromophenol (2,4,6-TBP)	118-9-6	330.8	4.9	0[a]	6.04	4.13[b]

[a] Average k not statistically different than zero.
[b] Howard and Meylan (14)
[c] Calculated by EPA Kowwin

passed student t-test with p-values < 0.001) with the exception of 2,4,6-TCP and 2,4,6-TBP, which failed the student t-test (i.e., p-values > 0.05). Suppressed uptake of 2,4,6-TCP and 2,4,6-TBP was exhibited even though plants were not inhibited. Contaminant profiles for 2,4,6-TCP and 2,4,6-TBP are shown in Figure 4.20 and demonstrated initial rapid contaminant equilibration with plant surfaces but no continued contaminant uptake, as demonstrated by constant plateau concentrations. Contaminant concentration profiles for exposure of active *L. minor* to 2,4,6-TCP and 2,4,6-TBP were typical of inactivated plant controls for 2,4,5-TCP reported in previous sections . Contaminant uptake rate coefficients for 2,4,6-TCP and 2,4,6-TBP were considered to be exceptionally low or zero uptake occurred. For the purposes of the analysis herein, k values for 2,4,6-TCP and 2,4,6-TBP were presented as zero because continued contaminant assimilation was not observed and first order rate coefficients were not statistically different from zero.

Effect of varying halogen substituents on contaminant uptake rate was examined. It is clear by inspection of example data in Figures 4.16 – 4.20 that substituent type and positioning affect rate of contaminant uptake. Concentration profiles of three tri-halogenated phenols (2,4,5-TCP, 2,3,5-TCP and 2-Cl-3,5-DFP) are presented in Figure 4.16. Rates of contaminant uptake were different for all three tri-substituted phenols presented in Figure 4.16, with statistically distinct k values. Comparing tri-substituted phenol data in Figure 4.16 with data for 2,4,6-trihalogenated phenols in Figure 4.20 indicated a wide range of uptake rates for various tri-substituted phenols examined. Rate of contaminant uptake was observed in the following order: 2,4,5-TCP > 2,3,5-TCP > 2-Cl-3,5-DFP >> 2,4,6-TCP = 2,4,6-TBP. Contaminant uptake data for four di-halogenated

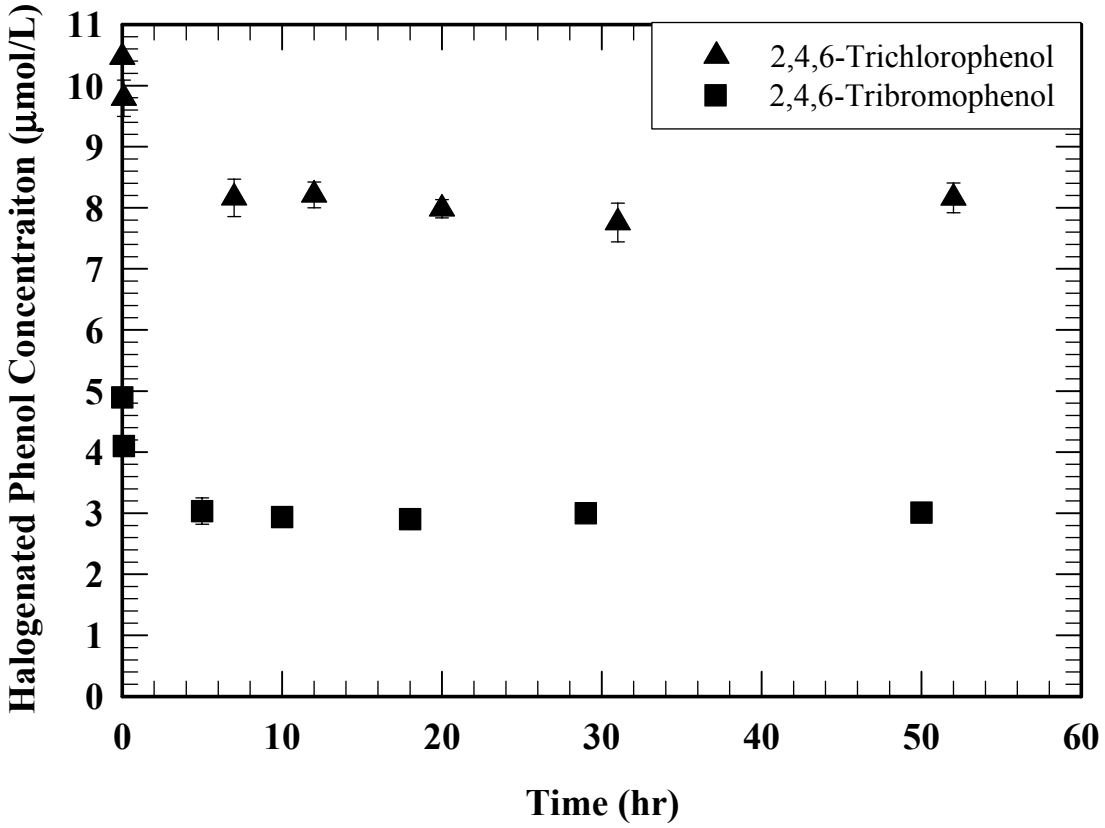


Figure 4.20. Contaminant uptake rates provided for *L. minor* exposure to 2,4,6-trichlorophenol and 2,4,6-tribromophenol. Plants retained constant plant activity throughout the course of the experiment, however continued contaminant uptake was not observed. First-order regression yielded rate coefficients that were not statistically different from zero.

phenols (2,4-DCP, 2,6-DCP, 4-Cl-2-FP, 2,6-DFP) are presented with k values in Figure 4.17. Contaminant uptake for di-substituted phenols in Figure 4.17 proceeded at relative rates of 2,4-DCP : 4-Cl-2-FP > 2,6-DCP : 2,6-DFP. Finally, data presented in Figure 4.18 represent contaminant concentration profiles for two di-halogenated phenols (4-CP and 4-FP), where contaminant uptake rates were similar, although not statistically the same.

A comparison of contaminant uptake rate coefficients (k) with varying halogen positioning is presented with corresponding log K_{ow} data in Figure 4.21. Data for three chlorinated phenols (2,4,6-TCP, 2,3,5-TCP and 2,4,5-TCP) are shown in Figure 4.21a, where k values ranged from 0 to 1.034 d^{-1} depending on substituent positioning. This wide variation in uptake rate for trichlorinated phenols was not explained by hydrophobicity as log K_{ow} values did not vary greatly and ranged only from 3.69 to 3.84. Similar results were observed for dichlorinated phenols (Figure 4.21b) where k values varied widely (0.292 – 0.927 d^{-1}) and little variation existed in log K_{ow} values (2.75 – 3.06). In addition, k values for mono- and dichlorinated phenols were within the range of k values observed for trichlorinated phenols (Figure 4.21a, 4.21b, and 4.21f) even though log K_{ow} values varied by 1.4 units between mono-, di- and trichlorinated phenols. No direct relationships existed between uptake rate and substituent positioning or substituent number and showed that rate of contaminant uptake by *L. minor* was not driven strictly by contaminant hydrophobicity.

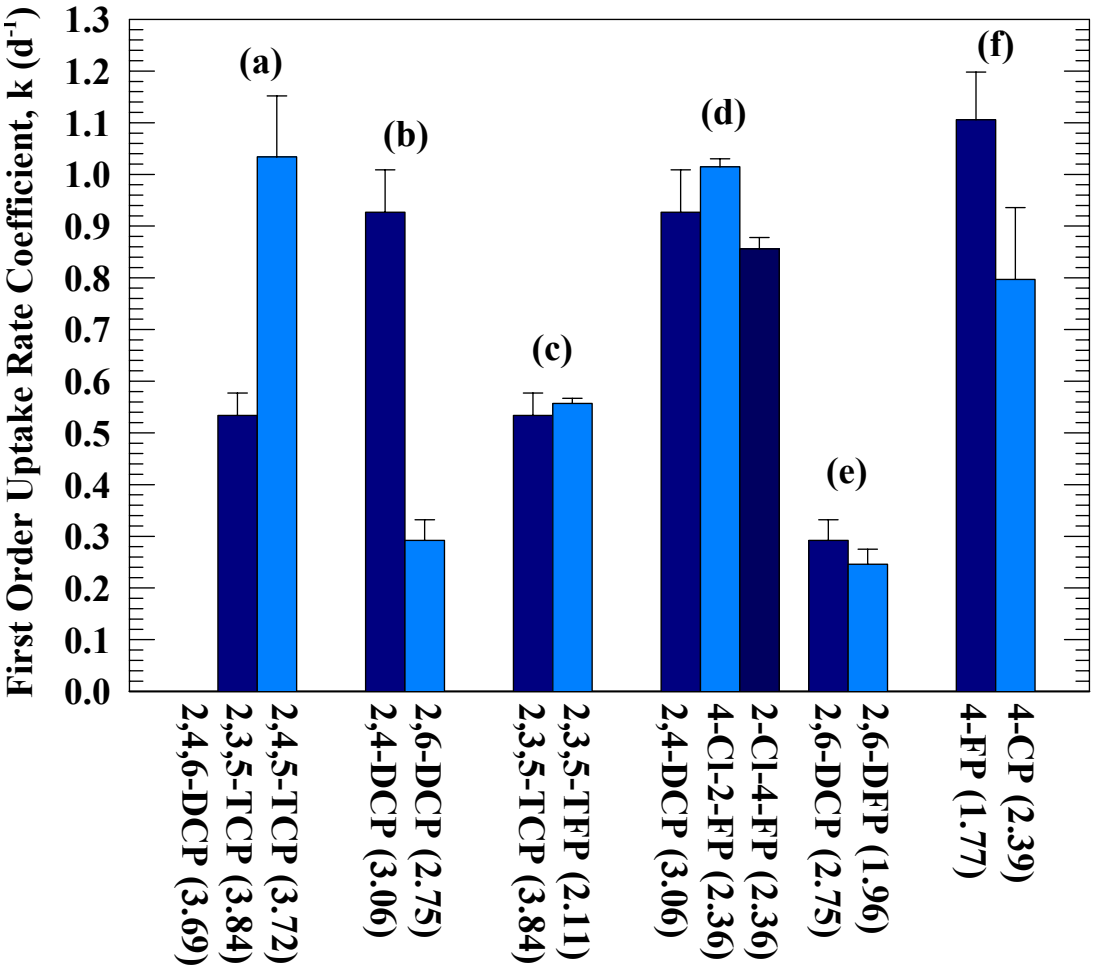


Figure 4.21. Wide variation were observed for k values measured for tri- and dichlorinated phenols (a, b) in spite of small variations in log K_{ow} values (given in parenthesis). Minimal variation was observed for k with substitution of fluorine for chlorine (c-f).

Substitution of fluorine for chlorine in a halogenated phenol resulted in a variable, but often minimal, change in contaminant uptake rate as was assessed via inspection of k values. No statistical difference existed between uptake rate of many fluorinated and chlorinated phenols even though log K_{ow} values typically decrease notably with substitution of fluorine for chlorine. For example, k-values for 2,3,5-TCP and 2,3,5-TFP were 0.534±0.043 $d^{-1}$and 0.551±0.010 d^{-1}, respectively, demonstrating no statistical difference in uptake rate even though log K_{ow} values differed by 1.7 units (Figure 4.21c). Rate of uptake of 2,4-DCP was not statistically different than 2-chloro-4-fluorophenol and 4-chloro-2-fluorophenol which were 0.927±0.082 d^{-1}, 1.015±0.015 d^{-1} and 0.856±0.022 d^{-1}, respectively (Figure 4.21d). In addition, no statistical difference was observed in k values between 2,6-DFP and 2,6-DCP, as demonstrated by k values of 0.246±0.029 d^{-1} and 0.292±0.040 d^{-1}, respectively, even though log K_{ow} values varied by 0.8 units (Figure 4.21e). However, a change in uptake rate was observed in some systems where chlorine was substituted for fluorine. For example, the k value for 4-CP (0.797±0.139 d^{-1}) was statistically different from the k value for 4-FP (1.106±0.092 d^{-1}) as is presented in Figure 4.21f. Examination of halogenated phenols uptake data demonstrated that effects of type of halogen substituted on contaminant uptake rate in aquatic plant systems are variable, but often minimal.

Relationships that might describe uptake of halogenated phenols by aquatic plants were investigated by comparing k values with physicochemical parameters relevant to partitioning processes and chemical structure were investigated. No significant relationships existed between uptake rate and molecular properties such as molecular

weight or molar volume. A comparison of molecular weight with k values is presented in Figure 4.22, where no relationship is apparent. Physicochemical parameters typically used to describe organic-carbon partitioning such as, log K_{ow}, log K_{oc} and molecular connectivity indices did not describe the rate of halogenated phenol uptake by aquatic plants. Data in Figure 4.23 compare k values with log K_{ow} values, and no significant relationship is observed (further discussion of k vs. K_{ow} comparison is provided is subsequent sections). Examples of k values compared with two molecular connectivity indices ($1\chi^v$ and $2\chi^v$) which can be used in linear relationships to predict hydrophobicity are provided in Figures 4.24 and 4.25 (Jorgensen *et al.,* 1998). No relationship between k values and $1\chi^v$ or $2\chi^v$ or any other molecular connectivity index were evident. Rate of halogenated phenol uptake by aquatic plants was not well described by molecular properties, substituent parameters or physicochemical parameters typically used to describe organic-carbon partitioning; therefore relationships related to rate of enzymatic processing of contaminants were explored.

Comparison of Halogenated-Phenol Uptake with Literature Predictive Relationships

Previous studies developed predictive relationships for uptake of organic contaminants by terrestrial plants (Briggs *et al.,* 1982; Burken and Schnoor, 1998) and projected the concept that plant processes have a limit with respect to bioavailability due to hydrophobicity. Researchers developed 3-parameter Gaussian curves using non-linear regression to relate the transpiration stream concentration factor (TSCF) to log K_{ow}. Burken and Schnoor (1998) examined 12 compounds of interest at hazardous waste sits,

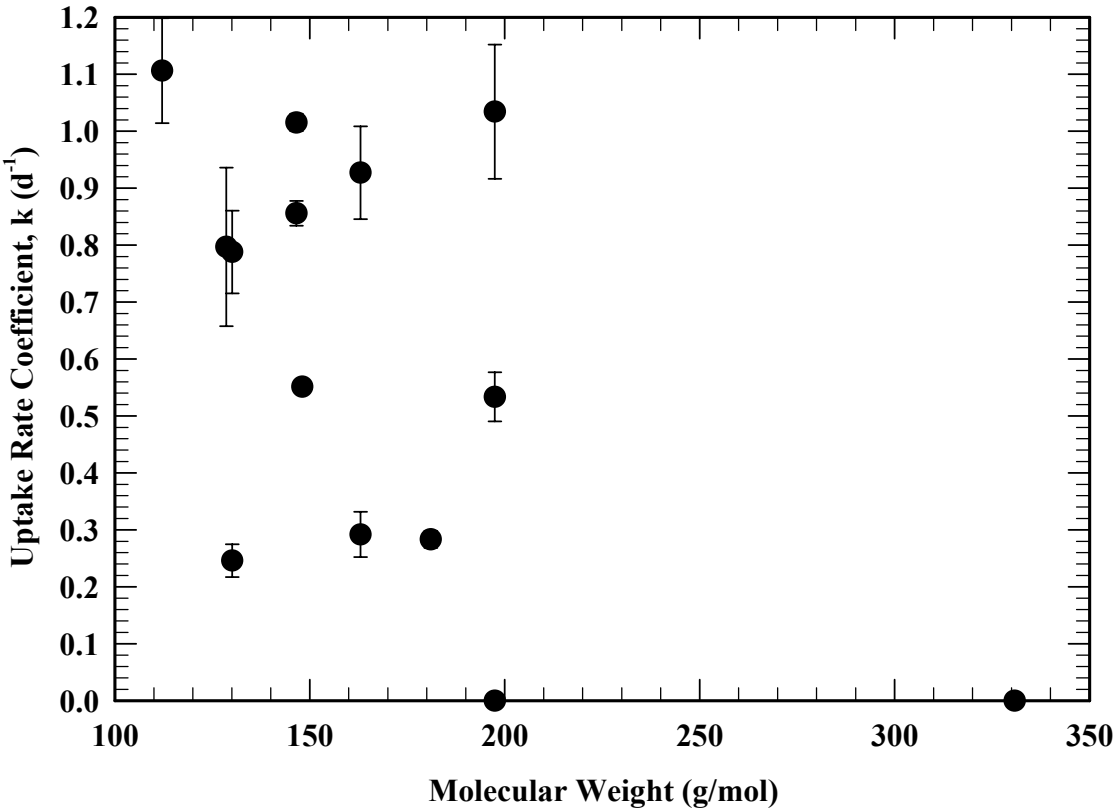


Figure 4.22. Comparison of contaminant uptake rate coefficient (k) with molecular weight.

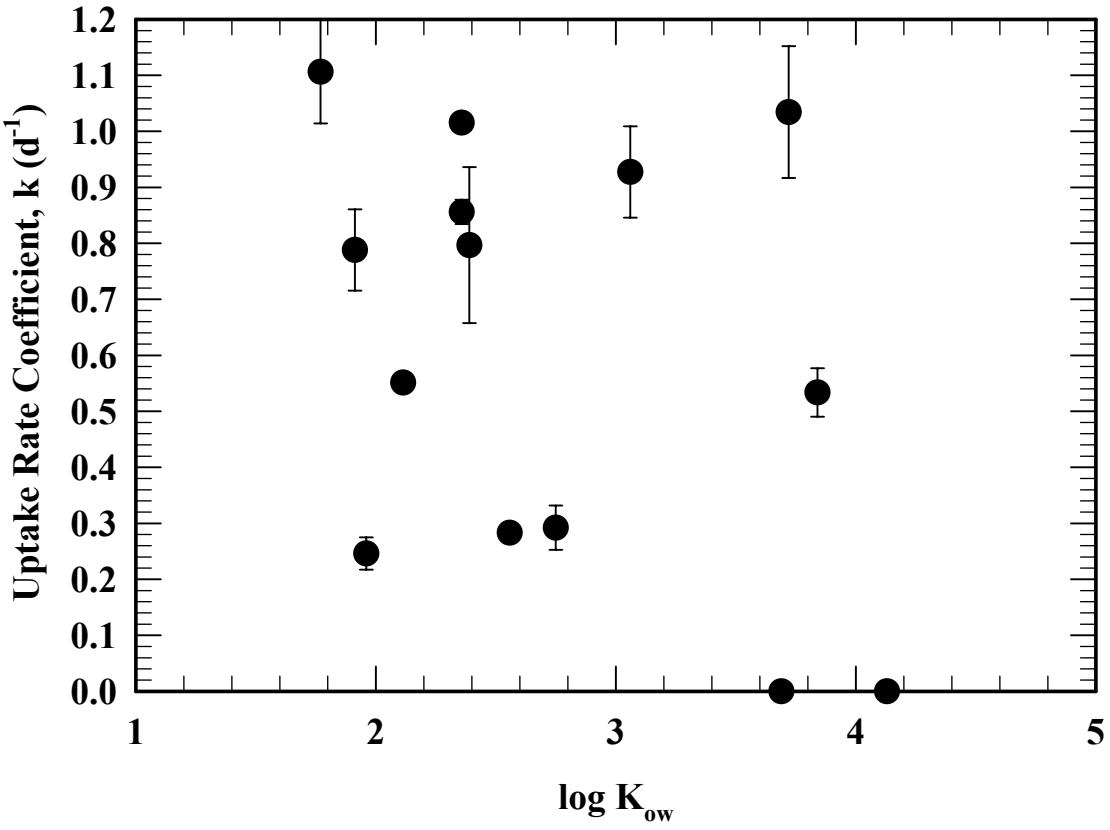


Figure 4.23. Comparison of contaminant uptake rate coefficient (k) with log K_{ow} values.

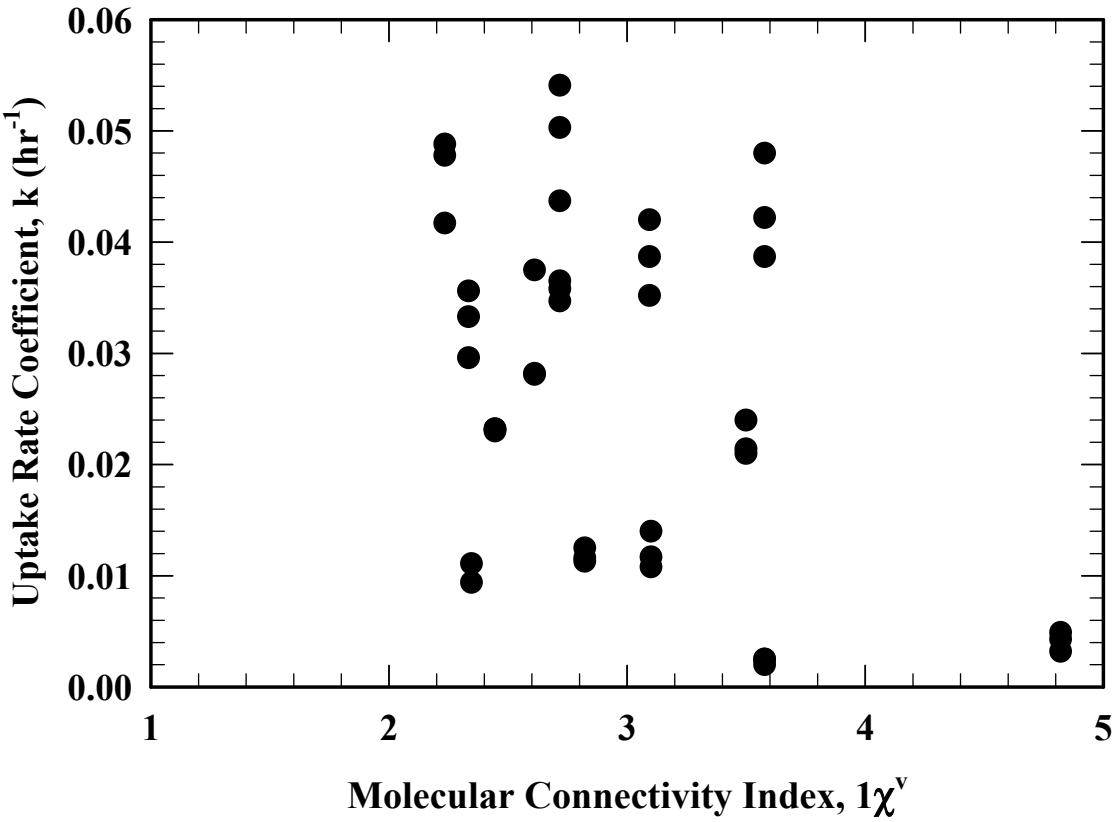


Figure 4.24. Comparison of contaminant uptake rate coefficient (k) with $1\chi^{v}$ molecular connectivity index.

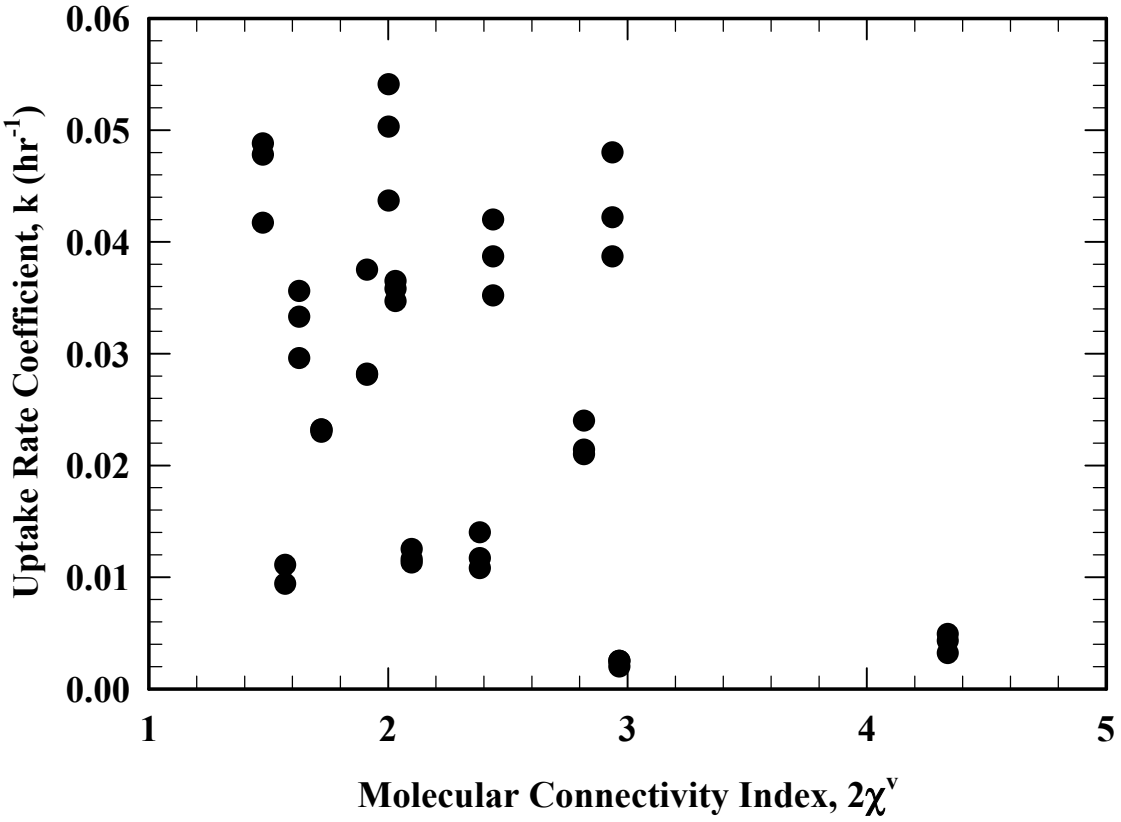


Figure 4.25. Comparison of contaminant uptake rate coefficient (k) with $2\chi^{v}$ molecular connectivity index.

including both ionizable (phenol, $pK_a = 9.99$; pentachlorophenol $pK_a = 4.9$) and non-ionizable compounds (RDX, aniline, benzene, TCE, atrazine, toluene, ethylbenzene, m-xylene, and TCB) with log K_{ow} values of 0.88 – 5.1, spanning a range of hydrophobicity. Briggs and co-workers (1982) studied two classes of non-ionizable compounds, O-methylcarbamoyloximes and substituted phenylureas, with log K_{ow} values of -0.55 – 4.6. In order to assess applicability of previously developed Gaussian relationships to aquatic plant systems, data gathered for halogenated phenol uptake by *L. minor* were compared to previously generated predictive relationships that related TSCF to log K_{ow}. Values of log K_{ow} for halogenated phenols examined were 1.77 – 4.13 and fit into previously used ranges of log K_{ow}. Additionally, media pH was fixed at a value of 5 which eliminated aqueous phase ionization as a factor related to partitioning into the plant. In comparison, Burken and Schnoor (1998) reported media pH values of 6.0 – 6.5 and Briggs and co-workers (1982) did not report pH of media.

TSCF can be qualitatively compared with uptake rate coefficients (k values) because both represent overall contaminant removal from their respective reactors. Additionally, both are constant with time and concentration while dependent on plant species and contaminant examined. Calculation of TSCF is impractical for *L. minor* since it is an aquatic plant of simple morphology. In addition, *L. minor* sorbs its water and nutrients through the lower surfaces of fronds, further demonstrating the impracticality of using TSCF and RCF with *L. minor*.

Experimental data which compared TSCF with log K_{ow} are reproduced from Briggs and co-workers (1982) and Burken and Schnoor (1998) in Figure 4.26. The relationship between TSCF and log K_{ow} produced a peak shaped curve, where contaminants with mid-range log Kow values were found in highest concentrations in the transpiration stream and contaminants with high or low log K_{ow} values were not readily translocated in plants. Researchers fit Gaussian curves to experimental data which generated peak values of 1.78 and 2.50 (Briggs *et al.,* 1982 and Burken and Schnoor, 1998, respectively) and plots of equations provided are shown in Figure 4.26.

Gaussian curves which related log K_{ow} to TSCF were qualitatively compared with log K_{ow} values for halogenated phenols and corresponding k values provided herein (Figure 4.27). Examination of data demonstrated that a range of k values (0 – 1.16 d^{-1}) occurred for high log K_{ow} values (i.e., log K_{ow} >3.5). Low k values (i.e., k = 0) observed for high log K_{ow} were consistent with previously generated Gaussian curves where TSCF values decreased rapidly for log K_{ow} > 3.5. However, high k values were also observed for log K_{ow} > 3.5, a result which was inconsistent with a Gaussian shaped curves and previous results. Similarly, a range of k values (0.23 – 1.17 d^{-1}) was observed for mid range log K_{ow} values (i.e., 1.91 > log Kow > 3.06) where peak TSCF values were observed by Burken and Schnoor (1998) and Briggs and co-workers (1982). Low k values observed for mid range log K_{ow} values were inconsistent with Gaussian shaped curves presented for terrestrial plant systems. No peak shape occurred in data gathered for halogenated phenols and results for halogenated uptake by *L. minor* were not consistent with hydrophobicity limited contaminated uptake. Therefore, it is reasonable to conclude that

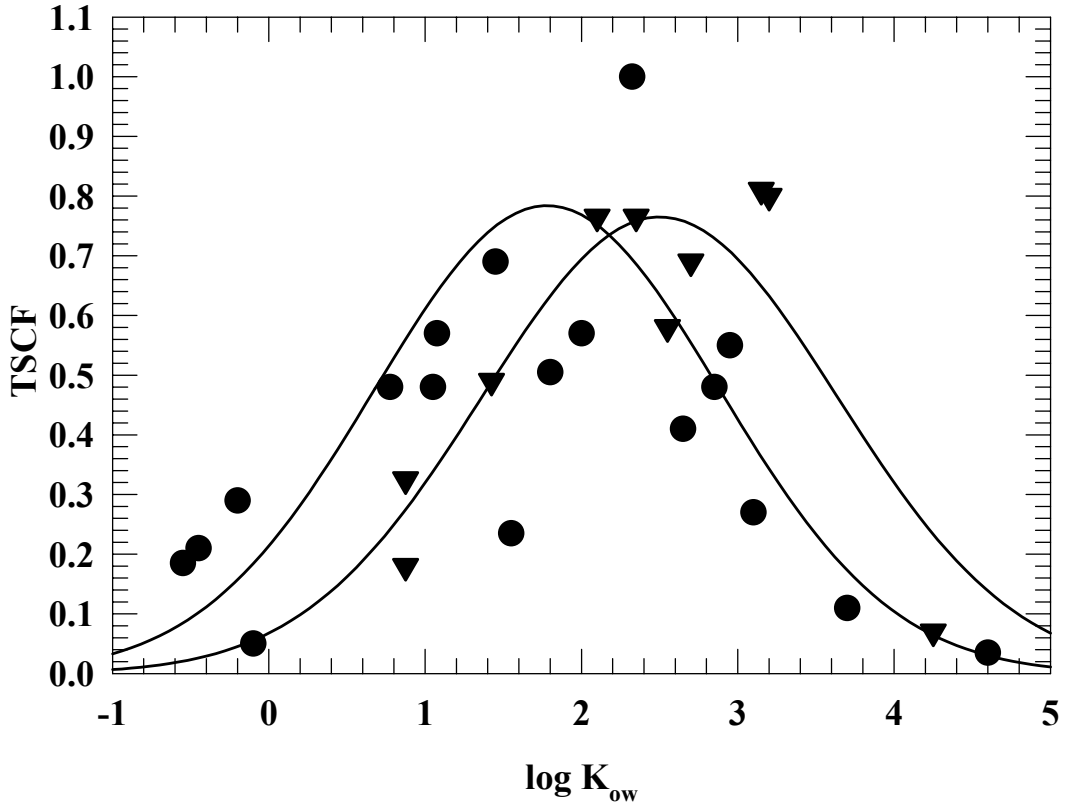


Figure 4.26. Predictive relationships to describe uptake of organic compounds by terrestrial plants were provided by (●)Briggs and co-workers (1982) and (▼) Burken and Schnoor (1998). Data taken from the literature relate TSCF with log K_{ow} for terrestrial plants and a variety of contaminants and herbicide compounds. Curves plotted represent predictive relationships provided from nonlinear regression of experimental data.

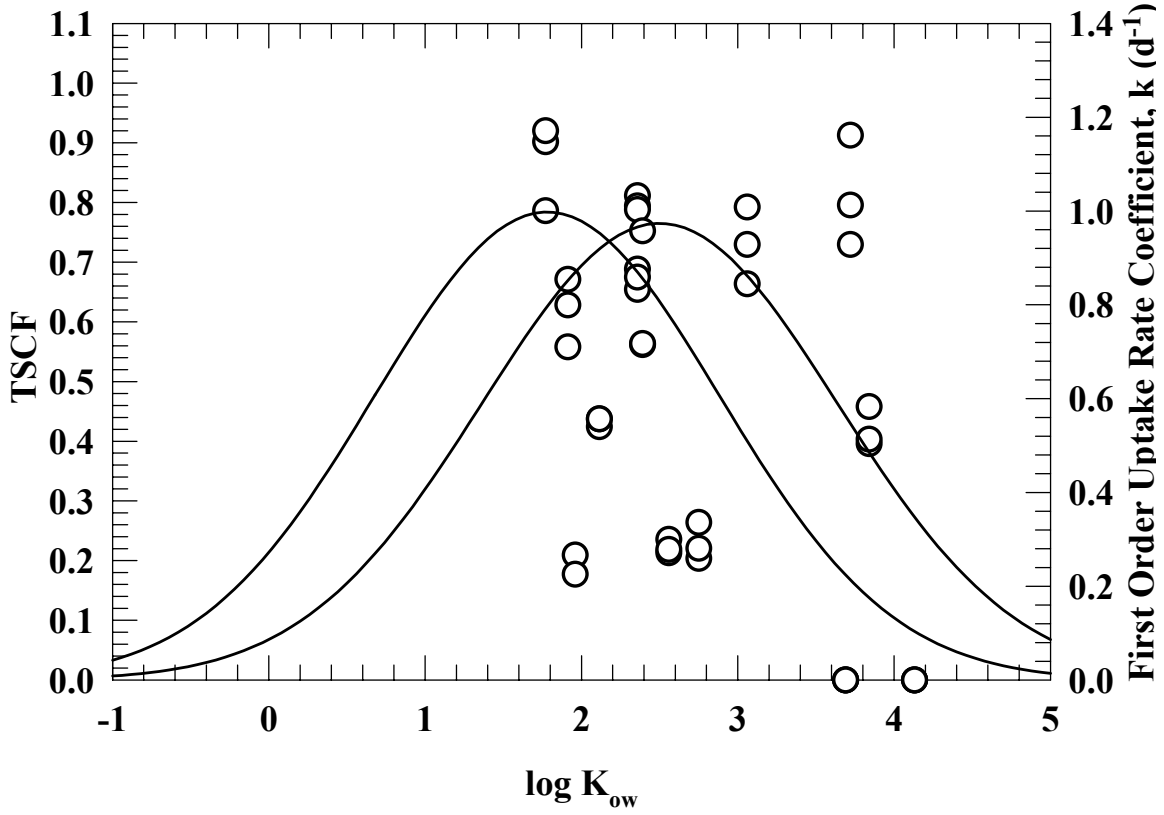


Figure 4.27. First-order rate coefficients for halogenated phenol uptake by *L. minor* (○) are compared with Gaussian relationships provided by Briggs and co-workers (1982) and Burken and Schnoor (1998). Curves plotted represent predictive relationships provided by Briggs and co-workers (1982) and Burken and Schnoor (1998) from nonlinear regression of experimental data presented in Figure 4.26.

data gathered for uptake of halogenated phenols by *L. minor* did not follow Gaussian relationships provided by Briggs and co-workers (1982) and Burken and Schnoor (1998). Attempts to fit halogenated phenol data to Gaussian and linear curves generated R^2 values of 0.098 and 0.20, respectively. The comparison of halogenated phenol uptake data with literature predictive relationships demonstrated that previously developed predictive relationships do not apply to halogenated phenol uptake by *L. minor*.

Effect of Speciation Internal to Plant on Contaminant Uptake Rate

No relationships were evident between contaminant uptake rate and parameters typically used to represent rate of partitioning into plants (i.e., parameters indicative of contaminant hydrophobicity or chemical structure such as log K_{ow}, log K_{oc}). Research investigating effects of plant activity and inhibition on contaminant uptake rate (presented in subsequent chapters) demonstrated that rate of contaminant metabolism internal to plants was a critical factor in determining contaminant uptake rate. Therefore, physicochemical parameters related to rate of contaminant metabolism internal to plants were considered in predictive relationships to describe rate of contaminant uptake by aquatic plants. Speciation of contaminants was considered as a factor driving rate of contaminant uptake by plants.

Contaminant uptake rate coefficients, k, were compared with pK_a values and a shifting-order pattern was apparent (Figure 4.28). A relationship existed where k increased linearly with increasing pK_a values in the range of 6.04 to ~8. A plateau value for k was observed for all contaminants with pK_a values greater than 8.0 where increasing pK_a

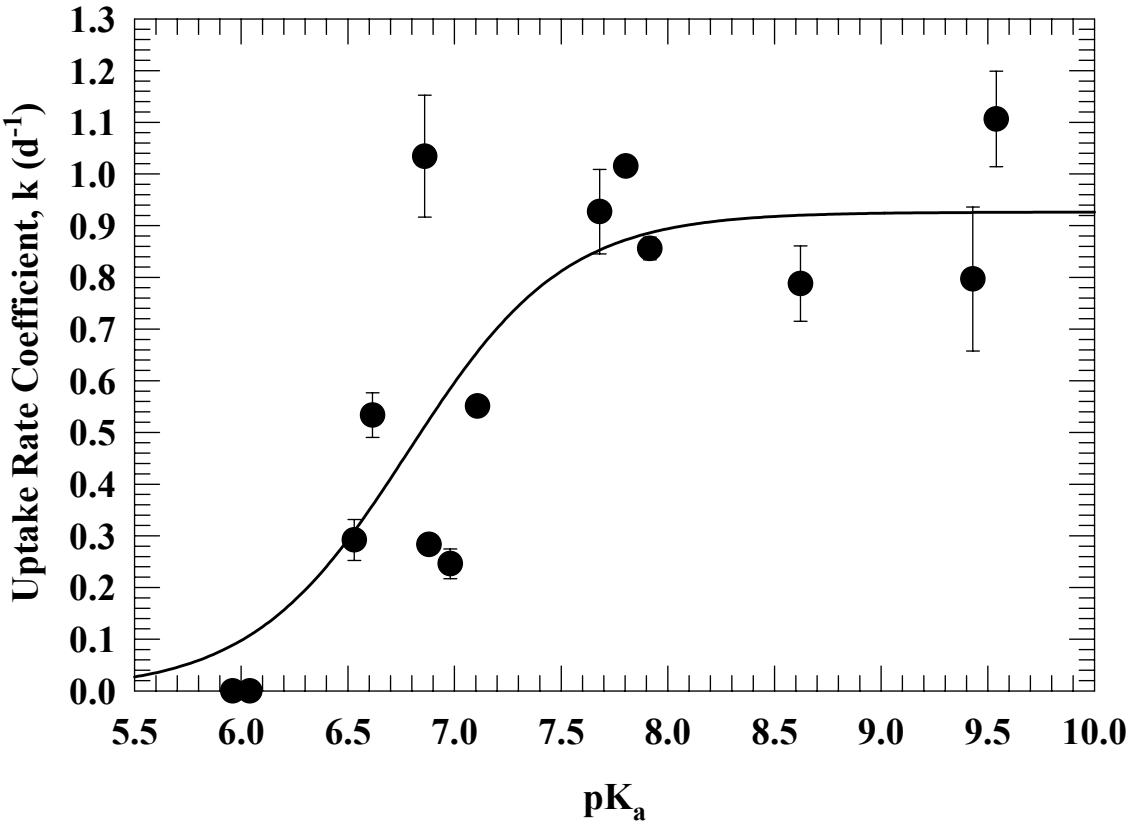


Figure 4.28. A linear relationship between pK_a and k was observed in the range of 6.04 to ~8.0. No further increase in k was observed for pK_a values greater than 8.0 and k values reached an average plateau value of 0.93 d^{-1}.

values did not further increase k values. These results indicated a relationship between contaminant speciation and k values where variations in contaminant speciation ceased at pK_a values greater than 8.0.

Discussion

Inactivated plant and photolytic controls demonstrated that contaminant removal was a live-plant mediated process, where losses due to photolytic degradation and accumulation due to water vaporization were considered to be negligible. Contaminants sorbed to surfaces of plants, and a Freundlich isotherm was used to quantify sorption to plant surfaces. Contaminant uptake occurred in live plants systems, and was demonstrated using *L. minor* and *M. spicatum*. Contaminant uptake rate was quantified using first-order non-linear regression and the first-order rate coefficient, k, was used to quantify rate of contaminant uptake. Increase in plant mass was shown to have a notable effect on contaminant uptake and addition of solvent increased rate of contaminant uptake. Photoperiod did not have an impact on uptake of contaminants by *L. minor*.

Phenols containing various halogen substituents were examined to determine the effect of halogen type and position on contaminant uptake. Halogen substituent type was observed to have minimal effect on contaminant uptake rate and in many cases, no significant difference was observed when a chlorine substituent was replaced with fluorine. These results demonstrated that fluorinated analogs of chlorinated phenols can effectively be used as tracers in aquatic plant systems. In addition, it was established that

no clear relationship between contaminant uptake rate and number of halogen substituents existed. A range of k values were observed for the same number of halogen substituents in various positions on the phenol. No definitive pattern was apparent with substituent positioning, however, it was clear that when the 2 and 6 positions were halogenated contaminant uptake rate was significantly less than similar contaminants with a halogen in the 2 position and not the 6 position. Further, when the 2, 4 and 6 positions were substituted with chlorine or bromine, no uptake was observed.

Predictive relationships for contaminant uptake developed based on partitioning processes were examined for application in aquatic plant systems. Previous research (Briggs *et al.,* 1982; Burken and Schnoor, 1998) demonstrated that a Gaussian relationship between log K_{ow} and TSCF described uptake of contaminants by terrestrial plants. Authors stated that contaminant uptake in terrestrial systems was dependant on contaminant hydrophobicity. However, no correlation was observed between contaminant uptake rate coefficient (k) and log K_{ow} for aquatic plant uptake of halogenated phenols. In addition, related physicochemical parameters typically used to model physical partitioning processes (log K_{oc}, MCI, molecular weight) were not useful for predicting uptake of halogenated phenols by *L. minor*. It is recognized that complex quantitative structural activity relationships (QSAR), incorporating four or more parameters, could potentially have been developed to predict uptake of contaminant by aquatic plants. However, the focus of this study was to enhance the understanding of the relationship between contaminant structure and uptake and metabolism. Hydrophobicity, often deemed the most important parameter controlling contaminant uptake in terrestrial

plants, did not describe contaminant uptake by *L. minor*. Consequently, relationships developed for terrestrial plant systems cannot always be readily applied in aquatic plant systems.

Previous work noted effects of structural and electronic characteristics on the Michaelis-Menton half velocity constant (K_m) of rat glucouonyltransferase when changing the halogen substituent on 2-fluoro-4-halophenols (Soffers *et al.,* 1994). No correlation was observed between structural and electronic characteristics that focused on the oxygen moiety in the phenol (e.g., HOMO density on oxygen, net charge on oxygen) and measured K_m values. Results led authors to conclude that contaminant hydrophobicity was the parameter that varied with substituent due to increased accumulation of the phenol in the membrane environment. However, contaminant pK_a and speciation in cells were not examined as factors influencing contaminant conjugation.

Physicochemical parameters related to rate of contaminant metabolism by plants were examined to provide additional insight into dynamics of contaminant uptake by aquatic plants. Speciation of halogenated phenols examined is a variable at neutral pH values (pKa values varied from 6.04 – 9.54) therefore pK_a was examined as a possible parameter controlling rate of contaminant uptake. Contaminant uptake rate increased linearly as pK_a values increased from 6 to 8. No increase in k was observed with pK_a values greater that 8. The aqueous phase pH was fixed at 5.0 so that all halogenated phenols examined were predominantly in the protonated form; therefore, contaminant availability in aqueous phase was not a factor affecting uptake rate. Examination of these results in

conjunction with results detailing dependence of partitioning on protonated form of the contaminant are provided in subsequent chapters.

CHAPTER 5

TRANSFORMATION AND SEQUESTRATION OF CHLOROPHENOLS BY AQUATIC PLANTS

Processing of organic contaminants by aquatic plants was investigated to understand fate of contaminants in aquatic-plant systems. Sequestration in plant systems was investigated using radioactive carbon (^{14}C) as a tracer. ^{14}C was used to clearly delineate contaminant internal to plants from aqueous-phase parent material or aqueous-phase metabolites. Transformation of organic contaminants was investigated using fluorinated analogs of chlorinated phenols as contaminant tracers. ^{19}F NMR was used to distinguish parent material from metabolites of fluorinated analogs internal to plants.

Sequestration of Contaminants by Aquatic Plants

The fate of 2,4,5-TCP and 2,4-DCP in aquatic plants *L. minor* and *M. aquaticum* was investigated using radiolabeled carbon on uniformly labeled aromatic rings.

2,4,5-Trichlorophenol Sequestration by *L. minor* and *M. aquaticum*.

Triplicate batch reactors were used to examine sequestration of 2,4,5-TCP by aquatic plants *L. minor* and *M. aquaticum*. *L. minor* was exposed to 2.7 mg/L 2,4,5-TCP and *M. aquaticum* was exposed to 4.4 mg/L 2,4,5-TCP, with pH in all reactors at a value of 8.

Reactors spiked with uniformly-labeled ^{14}C-2,4,5-TCP were examined in parallel with reactors not amended with radioactive carbon.. Radiolabeled reactors with *L. minor* contained 0.119 – 0.128 μCi of radioactive 2,4,5-TCP and radiolabeled reactors with *M. aquaticum* reactors contained 0.122 – 0.127 μCi of radioactive 2,4,5-TCP. Material recoveries were 104, 92, and 102% of the ^{14}C material at times 24, 48, and 120 hr, respectively, for *L. minor* and 94 – 96% for *M. aquaticum* indicating adequate material balance closures and radiolabel recovery. Experimental results indicated that *L. minor* rapidly removed 2,4,5-TCP from the aqueous phase. This concept was demonstrated by HPLC measurements of aqueous 2,4,5-TCP concentration in unlabeled reactors where 2,4,5-TCP concentrations were rapidly depleted (Figure 5.1). Aqueous measurements from parallel radiolabeled reactors indicated that radioactive material was depleted from aqueous phase at the same rate as aqueous 2,4,5-TCP was depleted from aqueous phase in unlabeled reactors (Figure 5.1). These results demonstrated that molecules of 2,4,5-TCP were removed from aqueous phase at the same rate as the carbon forming the aromatic ring in molecules of 2,4,5-TCP. Therefore, metabolites did not accumulate in media in significant quantities. Trace amount of the radiolabel tracer partitioned into the headspace (<2%) and a small amount of the tracer was found in the plant/reactor rinse water (<4%). Therefore, decreasing concentrations of 2,4,5-TCP in aqueous phase indicated that the base-molecule was being removed from the aqueous phase and sequestered in plant tissue.

Plants exposed to ^{14}C-2,4,5-TCP were oxidized to assess accumulation of 2,4,5-TCP in plant tissue. Activity measurement of oxidized plants were compared with aqueous

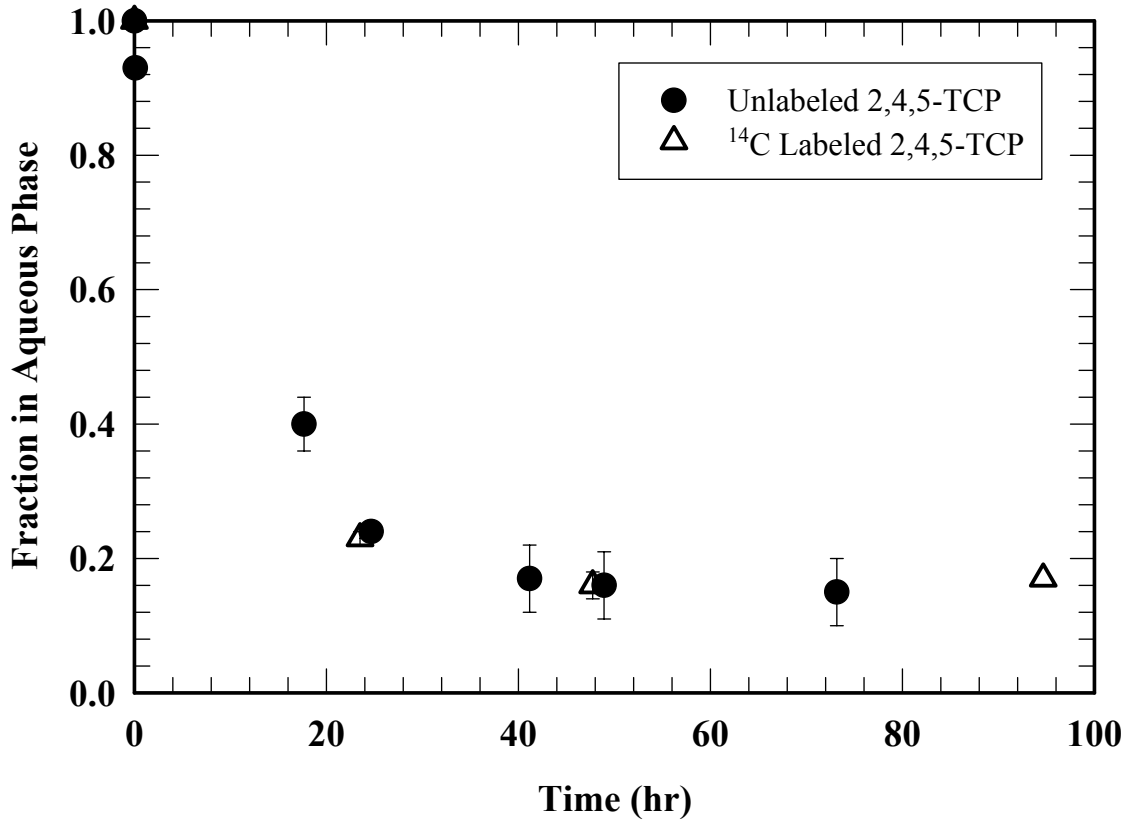


Figure 5.1. 2,4,5-TCP was removed from aqueous phase by *L. minor* in radiolabeled (^{14}C) and unlabeled reactors.

phase measurements of 2,4,5-TCP to determine if contaminant depleted from aqueous phase was accumulated in plant tissue. 2,4,5-TCP removed from aqueous phase in unlabeled reactors (i.e., $[2,4,5\text{-TCP}]_0 - [2,4,5\text{-TCP}]_t$) was compared with ^{14}C tracer accumulation within plant material in Figure 5.2. Removal of 2,4,5-TCP from the aqueous phase occurred in parallel with ^{14}C accumulation within the plant.

Results for unlabeled and radiolabeled experiments are summarized in Table 5.1, where fraction of 2,4,5-TCP remaining in aqueous phase, rinse, headspace and plant tissue was assessed at 24, 48 and 120 hr. As indicated in Table 5.1, trace quantities of ^{14}C were detected in headspace (< 2%). The pH of the aqueous phase was buffered to a value of 8, therefore, the observation of small quantities of tracer material in headspace did not necessarily indicate that the contaminant was mineralized. At a pH value of 8, inorganic carbon is found primarily as bicarbonate, thus is located in the aqueous media. Therefore, the trace ^{14}C in the headspace could be attributed to volatile impurities in the initial material.

Data for *L. minor* accumulation of 2,4,5-TCP shown in Table 5.1 indicate 76, 73 and 80% of the ^{14}C tracer was contained in the plant at 24, 48 and 120 hr. These data and high material recoveries (94 – 104%) demonstrated that virtually all of the contaminant removed from the aqueous phase was retained inside the plant. These data also indicated that contaminants accumulated in plants at significant concentrations. For example, after 48 hr, 2,4,5-TCP was concentrated at 90 μg 2,4,5-TCP/g *L. minor*. Because 2,4,5-TCP

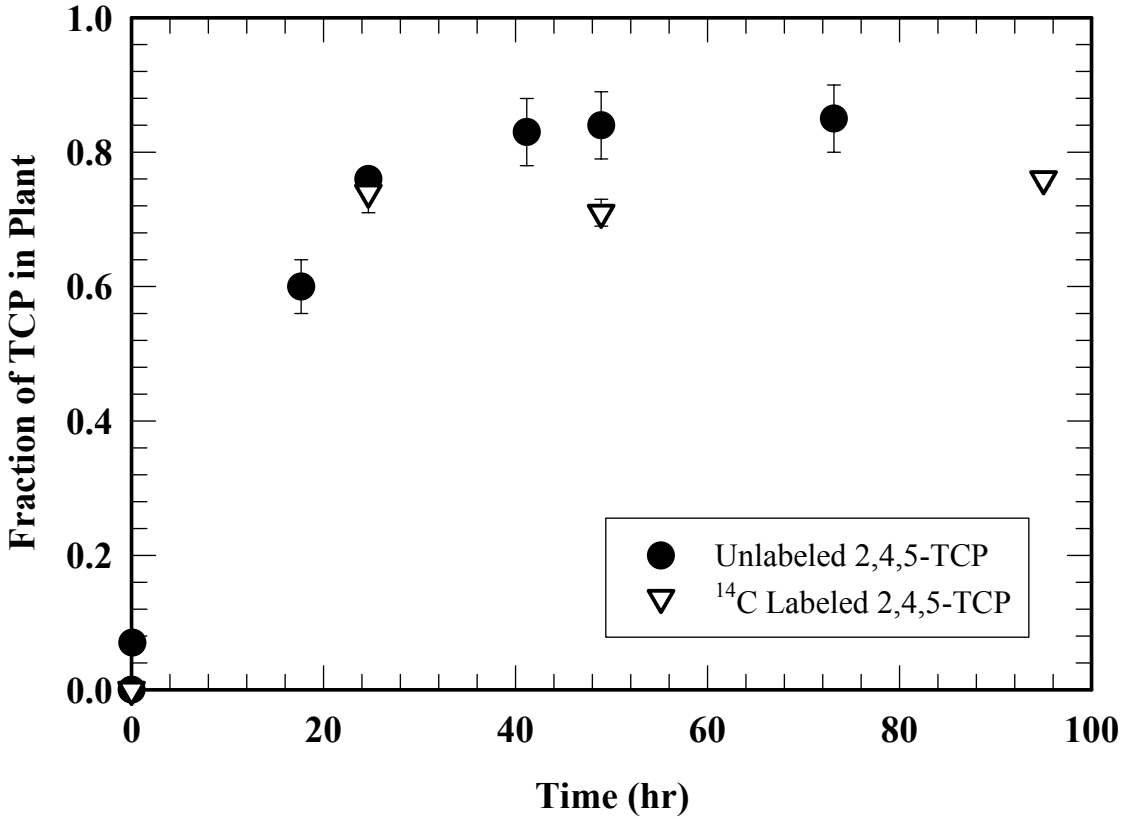


Figure 5.2. 2,4,5-TCP sequestered in *L. minor* based on net unlabeled 2,4,5-TCP that was removed from aqueous phase and ^{14}C-2,4,5-TCP sequestered in plant tissues.

Table 5.1. Material balance for ^{14}C-labeled 2,4,5-trichlorophenol in *L. minor* system.

	Material Detected 24 hr (%)	Material Detected 48 hr (%)	Material Detected 95 hr (%)
Aqueous Phase	23±1 (27±3)*	18±1 (19±6)*	18±0 (21±7)*
Plant / Reactor Rinse	4±1	2±0	2±1
Headspace	0±0	0±0	2±0
Plant	76±3	73±2	80±1
Material Recovery	104±4	94±3	102±0

* 2,4,5-TCP remaining in aqueous phase as determined by HPLC measurements

was accumulating within the plant, the uptake and sequestration was clearly a plant dependent process. Samples taken at 120 hr indicated that a small quantity of the radioactive tracer remained in the aqueous phase in both radiolabeled and unlabeled systems. 2,4,5-TCP was observed to be totally depleted from aqueous phase in many systems exposed to low concentrations, as described in Chapter 4. The 2,4,5-TCP remaining in aqueous phase in the systems presented in Figures 5.1 and 5.2 was attributed to inhibitory effects. Inhibitory effects were possibly a result of no headspace turnover and potential oxygen poisoning over 120 hr, carbon or nutrient limiting conditions or toxic effects due to 2,4,5-TCP.

Experimentation with *M. aquaticum* consisted of 17 g plant exposed to 4.4 mg/L 2,4,5-TCP. Results indicated significant 2,4,5-TCP removal from the aqueous phase in HPLC measurements of aqueous phase of unlabeled reactors (Figure 5.3). Radiolabeled reactors (amended with ^{14}C-2,4,5-TCP) showed removal of ^{14}C-2,4,5-TCP that was parallel to removal of 2,4,5-TCP in unlabeled reactors (Figure 5.3). ^{14}C-2,4,5-TCP was sequestered in plant material as was demonstrated by oxidation of the plant material as presented in Figure 5.4. Data for plant oxidation data were compared with contaminant removed from aqueous phase (i.e., $[2,4,5\text{-TCP}]_0 - [2,4,5\text{-TCP}]_t$), and 2,4,5-TCP removal from the aqueous phase paralleled accumulation of 2,4,5-TCP-associated carbon within the plant (Figure 5.4). Data for fraction of ^{14}C recovered in aqueous phase, plant material, headspace and reactor rinse are presented in Table 5.2. Trace ^{14}C material was found in the headspace (<2%) and in the plant rinse (<2%) and total mass recoveries were 91.7 – 98.6%.

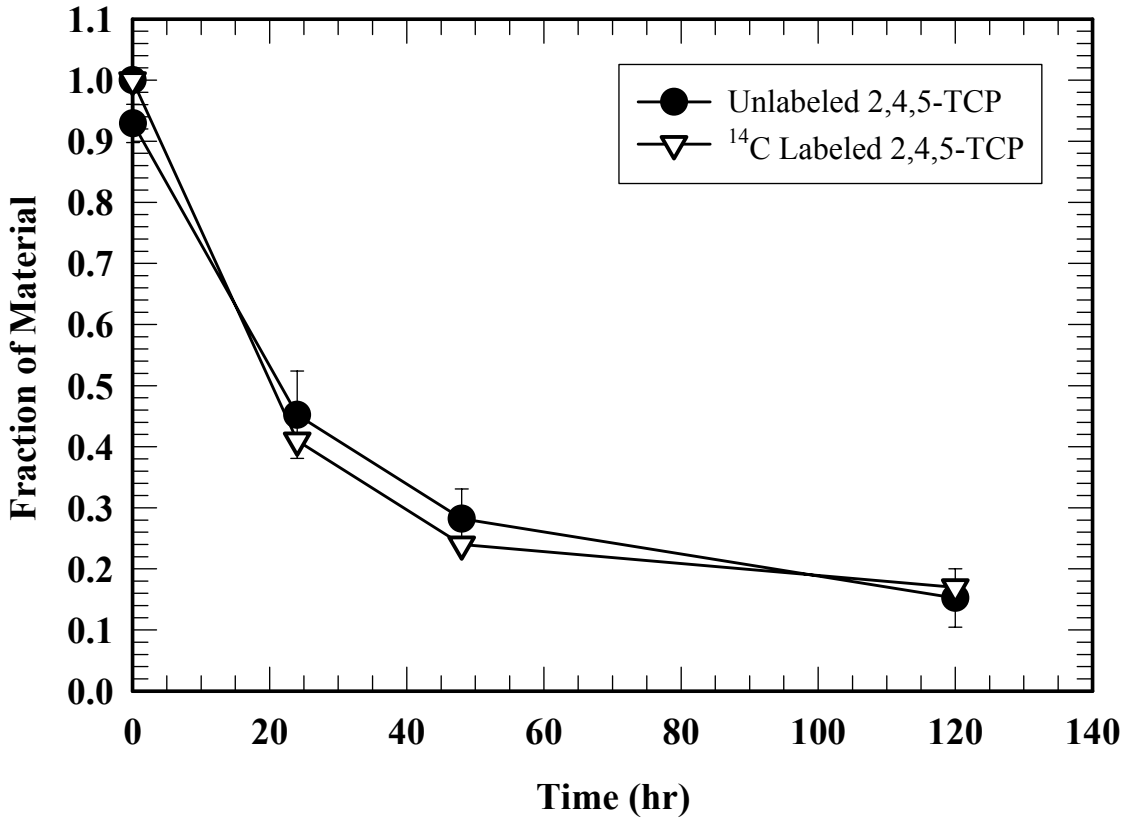


Figure 5.3. 2,4,5-TCP removed from aqueous phase by *M. aquaticum* in radiolabeled (^{14}C) and unlabeled reactors.

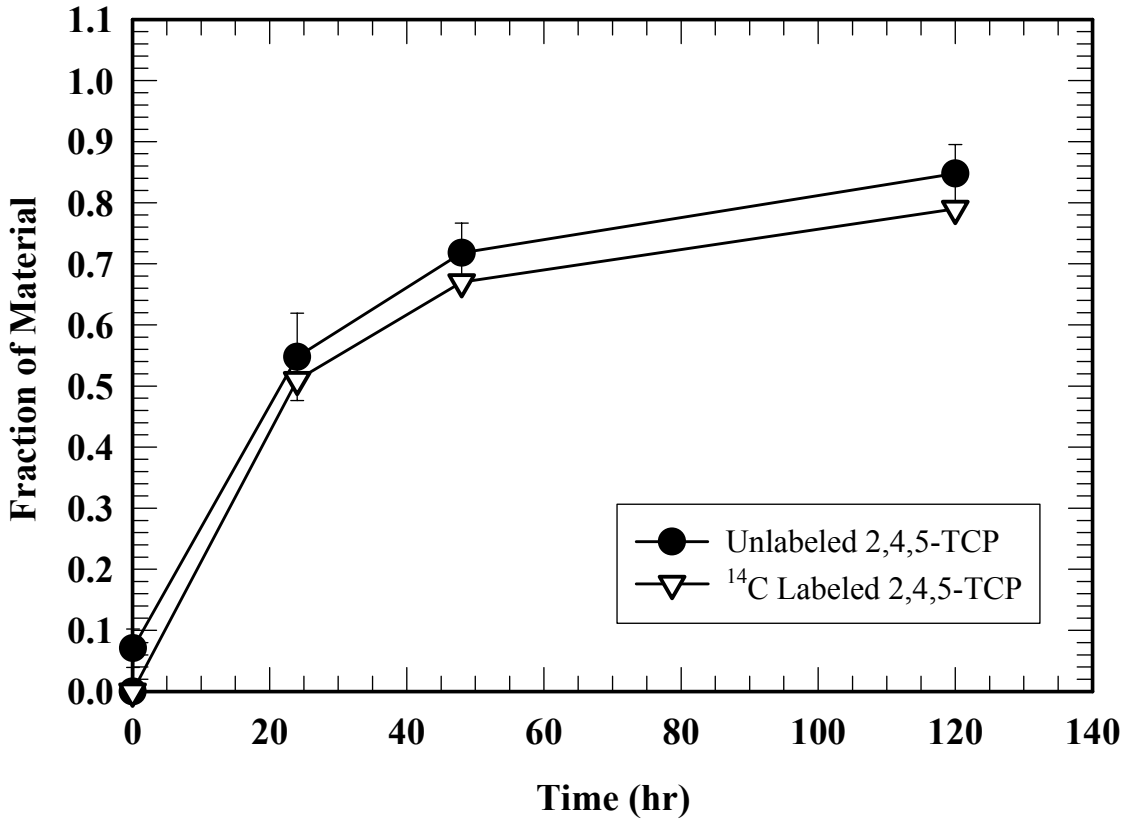


Figure 5.4. 2,4,5-TCP accumulated by *M. aquaticum* in radiolabeled (^{14}C) and unlabeled reactors.

Table 5.2. Material balance for ^{14}C-2,4,5-trichlorophenol in *M. aquaticum* system.

	Material Detected 24 hr (%)	Material Detected 48 hr (%)	Material Detected 120 hr (%)
Aqueous Phase	40.9 (45.2)*	23.8 (28.2)*	17.3 (15.2)*
Plant / Reactor Rinse	1.1	0.6	1.0
Headspace	0.5	0.6	1.5
Plant	51.1	66.6	78.8
Material Recovery	93.7	91.7	98.6

* 2,4,5-TCP remaining in aqueous phase as determined by HPLC measurements

Data showed significant accumulation of 2,4,5-TCP by *M. aquaticum*. Plants accumulated 51.1, 66.6 and 78.8% of 2,4,5-TCP in reactors at times 24, 48 and 120 hr. These data showed that 2,4,5-TCP was concentrated at the levels of 30, 39 and 46 μg/g plant at 24, 48 and 120 hr. The distribution of 2,4,5-TCP internal to *M. aquaticum* plants was assessed by separating plant material into roots, shoots and leaves and oxidizing each component. After 24 hr of exposure, significantly more ^{14}C-2,4,5-TCP was associated with roots (49%) than shoots (25%) or leaves (27%) as presented in Figure 5.5. After 120 hr of exposure, ^{14}C-2,4,5-TCP was evenly distributed between roots (34%), shoots (36%) and leaves (31%). These data demonstrate that 2,4,5-TCP was translocated within the plant, and accumulated in roots, shoots and leaves without preferential accumulation in specific component of plants. The contaminant therefore appeared to be uniformly distributed throughout the plant.

2,4-Dichlorophenol Sequestration by *L. minor*.

Sequestration of 2,4-DCP by *L. minor* was investigated using triplicate batch reactors. The system used 5 g *L. minor*, 200 mL of media at pH 8 and 2 mg/L 2,4-DCP. Reactors spiked with uniformly-labeled ^{14}C-2,4-DCP at concentrations of 0.018 – 0.190 μCi (radiolabeled reactors) were examined in parallel with reactors not amended with radioactive carbon (unlabeled reactors). Tracer recoveries were 97.1 and 104.1% of the ^{14}C material at times 24 and 48 hr, indicating adequate material balance closures and radiolabel recovery.

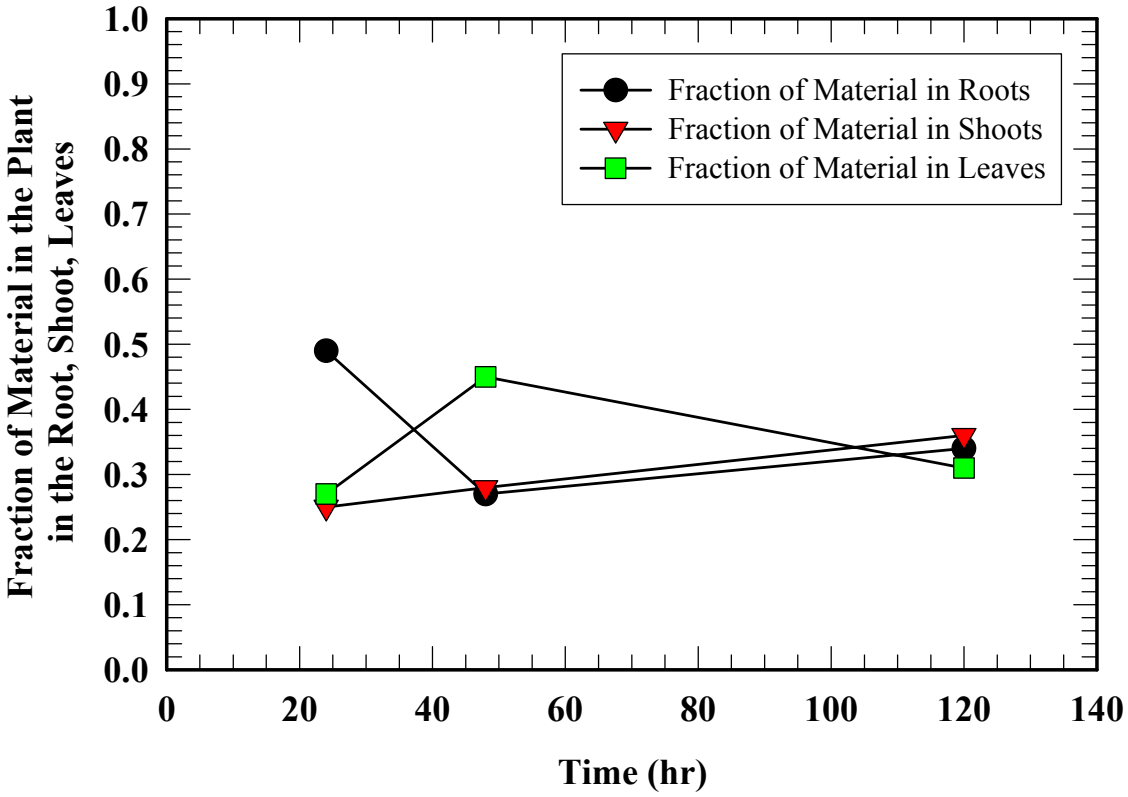


Figure 5.5. 2,4,5-TCP distribution in leaves, stems and roots of *M. aquaticum* as a fraction of ^{14}C located plants.

Rapid 2,4-DCP uptake by *L. minor* was established with HPLC measurements of aqueous phase in unlabeled reactors (Figure 5.6). Parallel radiolabeled reactors demonstrated that ^{14}C-2,4-DCP was taken up by plants at the same rate as 2,4-DCP in unlabeled reactors (Figure 5.6). Plant oxidation data demonstrated that significant quantities of 2,4-DCP were accumulated by plants. A comparison of ^{14}C accumulated by plants with 2,4-DCP removed from aqueous phase is presented in Figure 5.7 and results verified that the contaminant removed from the aqueous phase was sequestered in the plant material.

Data for fraction of ^{14}C material detected in aqueous phase, plants, reactor rinse and headspace are provided in Table 5.3. No radiolabel tracer partitioned into the headspace (0.0%) and a small amount of the tracer was found in the plant/reactor rinse water (<2.5%). Aqueous phase data indicated that only 22.0 and 8.3% of ^{14}C-2,4-DCP remained in aqueous phase after 24 and 48 hr of exposure, respectively. These data corresponded with aqueous phase concentrations determined using HPLC measurements which indicated 22.2 and 0.0% of aqueous phase contaminant remained after 24 and 48 hr, respectively. Data for plant oxidation indicated 72.9 and 93.7% of the ^{14}C tracer was contained in the plant at 24 and 48 hr. Results indicated *L. minor* rapidly took up and sequestered the carbon associated with 2,4-DCP and all of the 2,4-DCP removed from the aqueous phase was retained inside the plant.

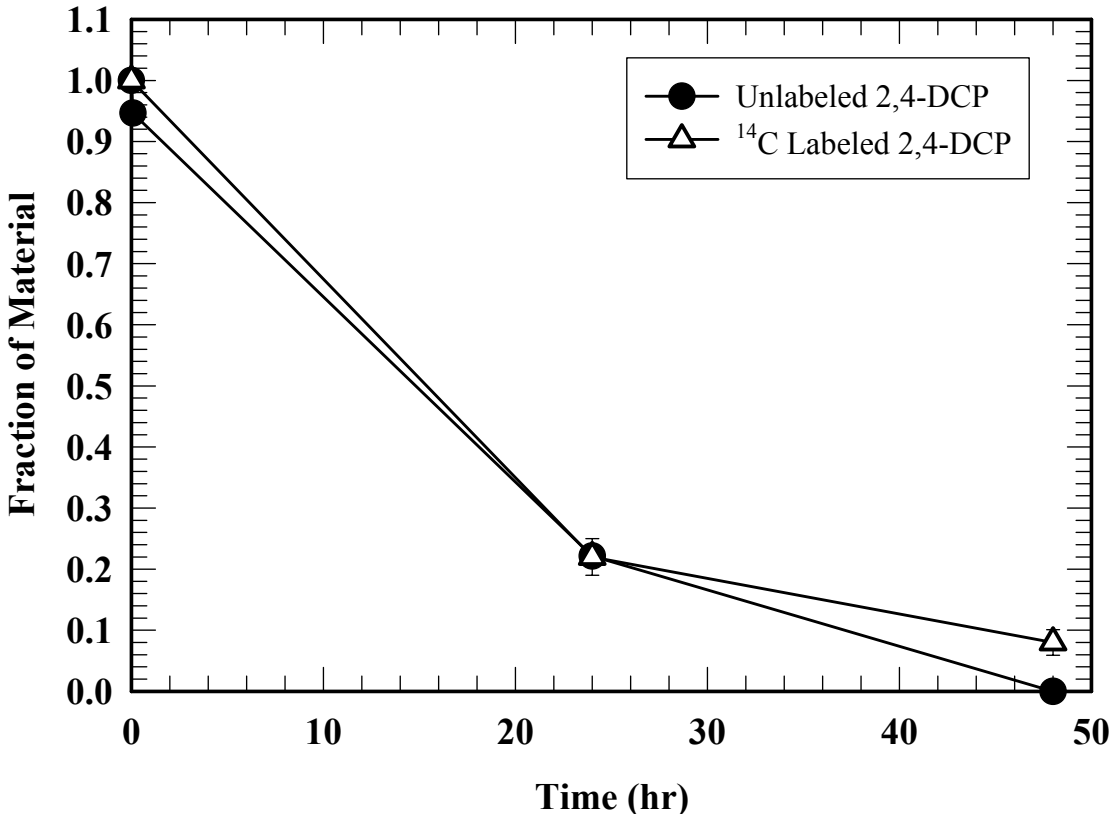


Figure 5.6. 2,4-DCP removed from aqueous phase in radiolabeled (^{14}C) and unlabeled reactors.

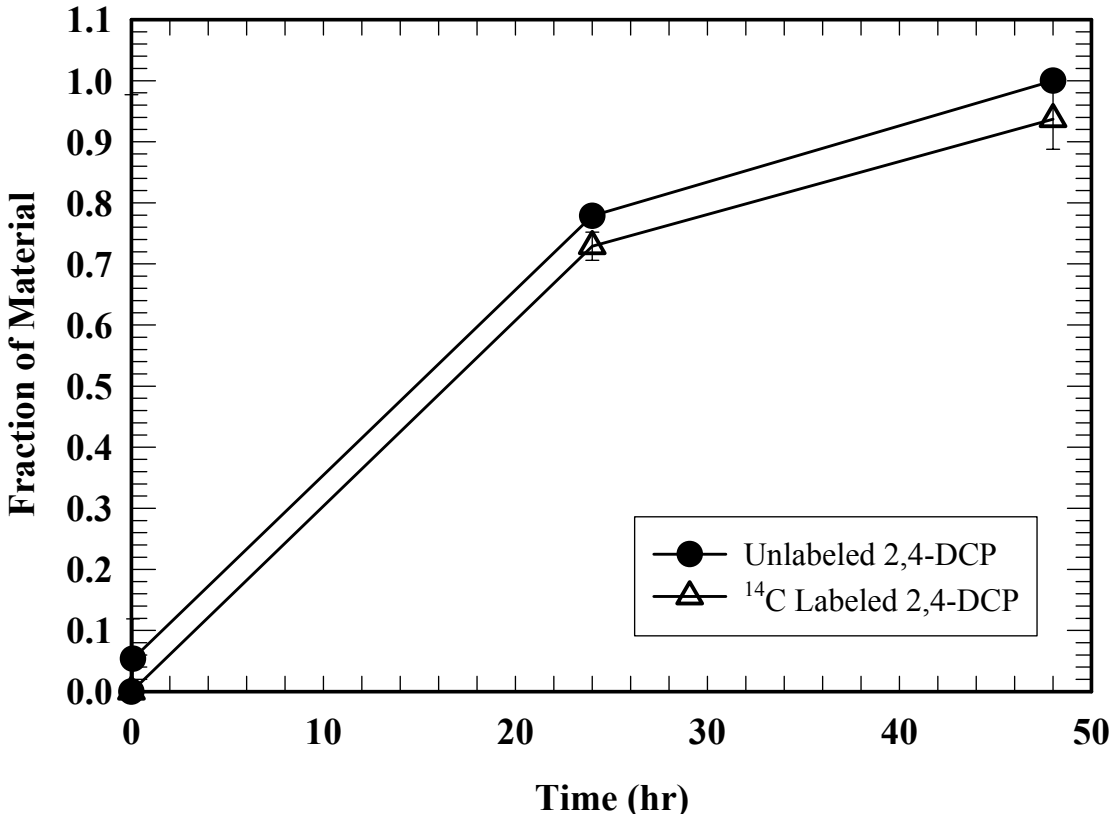


Figure 5.7. 2,4-DCP sequestered in *L. minor* as evidenced by ^{14}C-2,4-DCP accumulation in plants.

Table 5.3. Material balance for ^{14}C-2,4-dichlorophenol in *L. minor* system.

	Material Detected 24 hr (%)	Material Detected 48 hr (%)
Aqueous Phase	22.0±3.0 (22.2±0.6)*	8.3±2.1 (0.0±0.0)*
Plant	72.9±2.3	93.7±4.9
Plant / Reactor Rinse	2.1±1.3	0.8±0.0
Headspace	0.0±0.0	0.0±0.0
Material Recovery	97.1±2.1	104.1±6.2

* 2,4-DCP remaining in aqueous phase as determined by HPLC measurements

Measurement of Parent Compounds and Metabolic Products Sequestered in Plants Using ^{19}F NMR

Experimentation with radiolabeled materials demonstrated that chlorophenols were sequestered by aquatic plants *L. minor* and *M. aquaticum* with no release of parent material or metabolic products into the aqueous phase. This research was enhanced with delineation of contaminant fate in plant systems and information about sequestration of metabolic products and parent material internal to plants. A means of quantifying both parent materials and transformation products stored internal to plants was necessary. For purposes of examination of contaminant fate and metabolic product kinetics, classification of contaminant internal to plants in terms of chemical properties and metabolic processing phase (e.g., parent compound, conjugated materials, reduced materials, or oxidized materials) was considered to be a critical aspect of understanding fate of contaminants in plant systems. Therefore, classification of materials after the green liver model developed by Trapp and McFarlane (1995) with differentiation between Phase I, II and III products was desired rather than identification of specific chemical structures of metabolic products generated. Identification of contaminant-derived products sequestered internal to plants through routine techniques such as liquid and gas chromatography coupled with mass spectrometry is an intensive process that does not yield easily to quantification because of multiple extraction and sample clean-up steps. Complex derivatization is required for analysis using gas chromatography of semi-volatile compounds, an analytical technique which is inappropriate for use with large molecular weight and non-volatile moieties. Liquid chromatography often involves numerous clean-up procedures to avoid large molecular weight moieties typically found

in natural systems. ^{19}F NMR is a non-destructive technique which does not require compound derivatization or sample clean-up. Therefore, ^{19}F NMR will be used for rapid identification and quantification of metabolic products of contaminants.

Metabolite production can be tracked using ^{19}F NMR as a means of identifying and quantifying any compounds that are derivatives of a parent compound which retain the fluorine substituent. The chemical shift and intensity of an NMR spectrum are used to identify and quantify compounds thus establishing NMR as a technique that allows for identification of modifications in molecules and quantification of those modified molecules (Veeman, 1997; Rietjens, 1993). Quantification of materials is achieved through comparison of relative intensities of resonance lines which provides relative molar masses of nuclei of interest in a sample. Chemical shift is a sensitive means of differentiating between molecules where small changes in molecular structure numerous bond distances away from the nucleus of interest will cause a change in the resonance line. Information regarding bond interactions can be gained from the magnitude and direction of a chemical shift, and peak splitting provides information regarding interactions with nearby fluorine or hydrogen atoms.

Because natural fluorine containing organics are rare, interferences with NMR analysis would not be expected and all resonance peaks are attributable to fluorinated contaminants and metabolites. Additionally, ^{19}F NMR allows for simultaneous quantification of fluoride ions sequestered within the plant. These techniques will allow

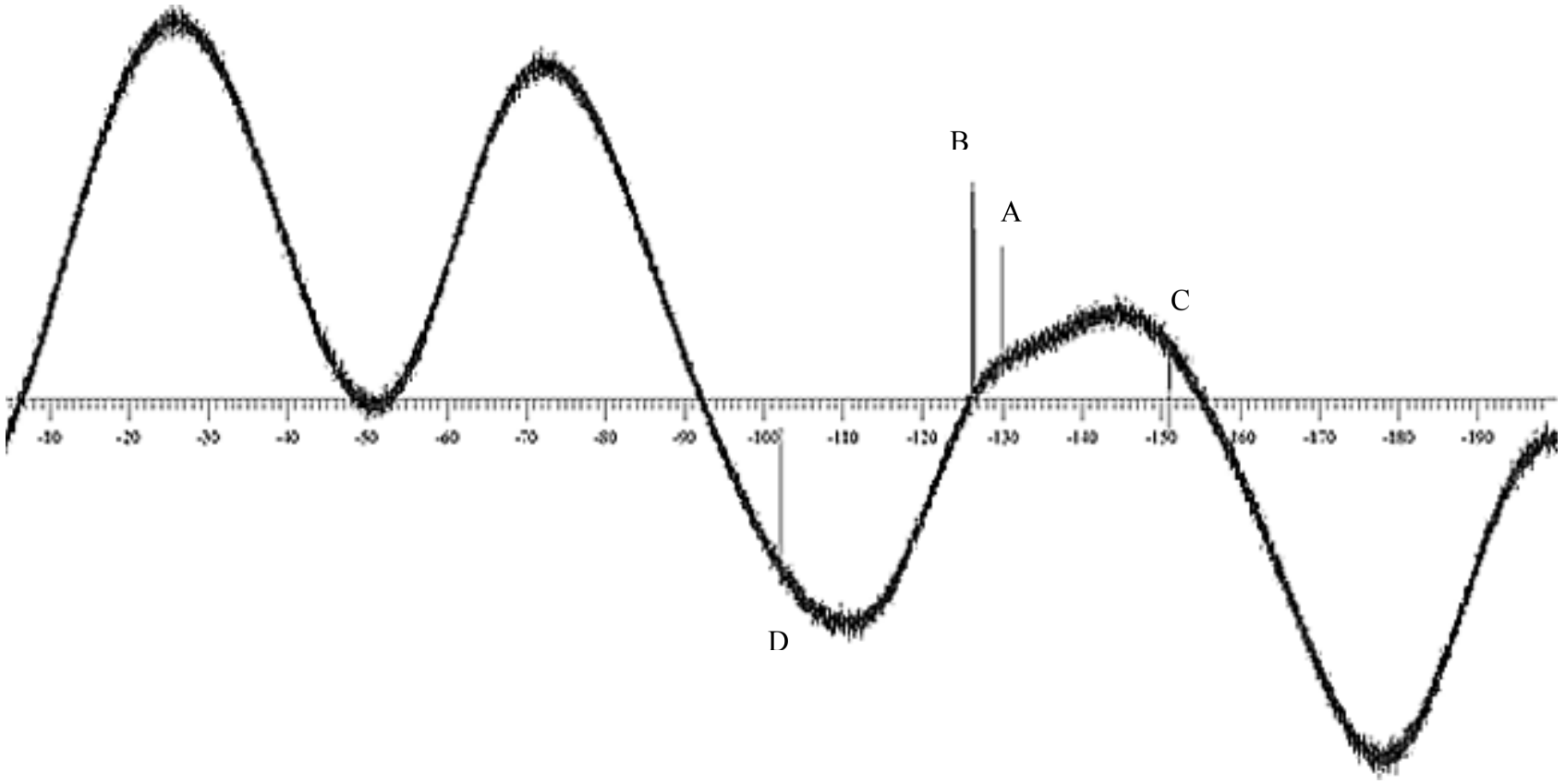
for quantification of contaminants and their complexation and degradation products in aqueous and plant phases.

Identification of Chlorophenols in Liquid Extracts via ^{19}F NMR

^{19}F NMR was used to detect and quantify chlorinated phenols internal to plants. Fluorinated analogs of chlorinated phenols were used with a focus on 4-chloro-2-fluorophenol (4-Cl-2-FP) as an analog of 2,4-DCP. Rate of uptake by *L. minor* for 4-Cl-2-FP was statistically the same as that of 2,4-DCP (Figure 4.17 and Table 4.2), therefore 4-Cl-2-FP was considered to behave similarly to 2,4-DCP in *L. minor* systems. Substituents in the ortho position in phenolic compounds exert a more significant effect on hydroxyl moiety speciation than meta or para substituents (Schwarzenbach, 1993). Therefore, an ortho positioned fluorine was considered to be most sensitive to conjugation or other changes dealing with the hydroxyl substituent.

Initial experiments conducted involved exposure of plants to aqueous 4-Cl-2-FP followed by sacrificing exposed plants and extracting compound of interest using acetonitrile extraction techniques described in Chapter 3. A ^{19}F spectrum was developed using a wide spectral window (>300 ppm) and a large number of scans (20,000 scans) in order to identify the number of metabolites present in the acetonitrile extracts and their chemical shift. Figure 5.8 presents a spectrum developed for an acetonitrile extract which contained four possible peaks labeled A through D, with chemical shifts -130.0; -126.0 to -126.5; -102.2; and -150.0, respectively.

Figure 5.8. Example of spectra gathered with a large spectral window (>300 ppm) and 20,000 scans.



Peak A (-130.0) was identified as the parent material (4-Cl-2-FP) sequestered internal to plants and an enlargement of peak A is presented in Figure 5.9. Identification of 4-Cl-2-FP was achieved by comparison of the chemical shift of the spectrum shown in Figure 5.9 to a spectrum of an acetonitrile extract of an unexposed plant which was spiked with 4-Cl-2-FP, where the peak comparison is presented subsequently in Figure 5.14. In addition to parent material and conjugated metabolites identified in Figure 5.8, two additional peak shapes (peak C and D) appeared in the large spectral-window spectrum used to gather data presented in Figure 5.8. Peaks C and D were confirmed as artifacts due to their narrow peak width and inconsistent appearance. No other peaks were present in the spectrum presented in Figure 5.8 or other wide-spectrum, numerous-scan spectrum verified that no other fluorinated metabolites of 4-Cl-2-FP were present in acetonitrile extracts of exposed plants.

Peak B (-126.0 to -126.5 ppm) was 3.5 ppm downfield of Peak A (-130.0), and it was apparent from the small chemical shift between Peak A and Peak B that the compounds were very closely related. The spectral window for ^{19}F NMR is ~500 ppm, therefore 3.5 ppm represents a small change in the chemical environment surrounding the fluorine nucleus (Veeman, 1997). It was established that materials detected in Peak B were multiple metabolic products of 4-Cl-2FP because all peaks observed were attributable to compounds of 4-Cl-2FP due to the absence of background fluorine in plant systems (Rietjens *et al.,* 1993). Resonance lines for both parent material and metabolites were near the range of fluorinated aromatics that have previously reported by Gordon and Ford (1972), which was -108 to -119 ppm. These results indicated that metabolic products

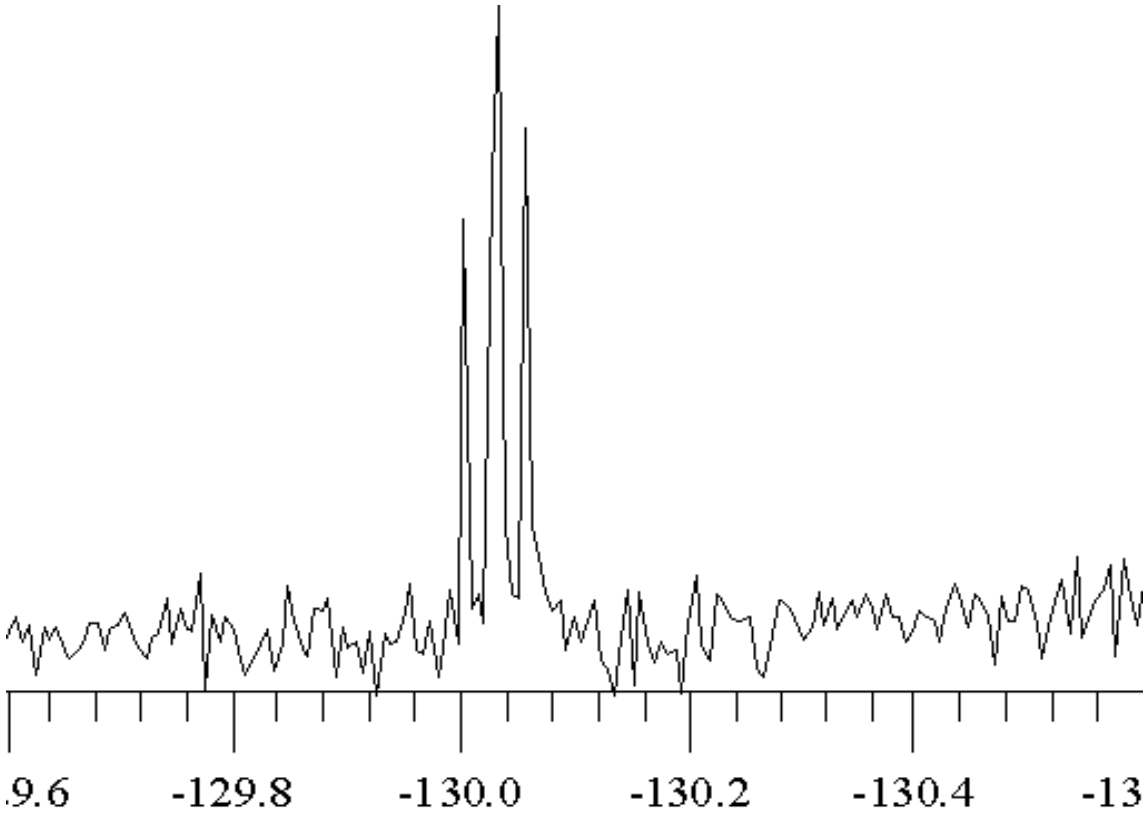


Figure 5.9. A portion of the spectrum shown in Figure 5.8 providing the detailed peak splitting of 4-chloro-2-fluorophenol.

observed in Peak B maintained the aromatic ring structure of the parent material. In addition, no peaks were observed in ranges where mono-substituted aliphatics were reported (205 to 225 ppm) indicating that ring cleavage did not occur (Gordon and Ford, 1972). The chemical shift of fluorine in acetonitrile extract matrix was –102.2 ppm through observation of an unexposed plant which was spiked with fluoride. It was established that the fluorine substituent was not cleaved from 4-Cl-2FP and sequestered in plants because no peaks were observed at –102.2 ppm.

Resonance lines for peak B were downfield of parent material in peak A. The relative positioning of the resonance lines of parent material and metabolites in the spectrum were derived from the fact that the fluorine nucleus in the metabolites was more shielded by the magnetic field of its surrounding atoms than the fluorine nucleus of the parent material. A nucleus undergoes increased shielding when the electron density surrounding it is increased, and a nucleus with increased shielding appears upfield of a nucleus with less shielding (Skoog *et al.,* 1998). This concept is consistent with conjugation of phenol in the metabolites causing a decrease in the electron-withdrawing capabilities of the hydroxyl moiety resulting in an increase in the electron density surrounding the fluorine nucleus and an increase in the shielding of the fluorine nucleus. This point is made further by examining the chemical shift of fluorobenzene, a fluorinated aromatic which does not contain a hydroxyl moiety. The chemical shift of fluorobenzene was -109.7 ppm, which was 20.3 ppm downfield of 4-Cl-2-FP due to increased shielding of the fluorine nucleus when no hydroxyl moiety was present to withdraw electrons from the aromatic ring.

Spectral details of plant-produced metabolites are presented in an enlargement of Peak B (Figure 5.10) which shows three or four peaks of closely related compounds. Peaks representing metabolites of 4-Cl-2FP are overlapping because of the similarity in chemical environment of the fluorine substituent for all compounds detected. Because the peaks were overlapping, it was impossible to distinguish the exact number of compounds with a chemical shift in the range of -126.0 to -126.5 ppm.

To gain additional information regarding number of metabolites present in acetonitrile extracts, additional wide spectrum data (Figure 5.11) were gathered using an instrument with a larger magnet (500 MHz, 11.7 T) than contained in the instrument (400 MHz, 9.4 T) used to generate data in Figure 5.8. Sensitivity of detection in changes of environment surrounding the fluorine nucleus increases with increasing magnetic strength of an instrument (Skoog *et al.,* 1998). The spectrum presented in Figure 5.11 (gathered using 500 MHz instrument) shows three large peaks in the range of -131.9 to -132.1 ppm which represented metabolites of 4-Cl-2-FP and the majority of the fluorine nuclei in the sample. Two or three smaller peaks appeared in the range of -131.4 to -131.9, which were very similar in chemical structure to those metabolites which existed in the range of -131.9 to -132.1 ppm, but were present in significantly less quantity. A single peak representing parent material appeared at -135.7 ppm. A chemical shift of ~3.5 ppm existed between parent material and metabolites, just as was observed in data shown in Figure 5.8. The increased resolution in Figure 5.11, which resulted from increased magnet size, showed that many metabolites (~6) with similar chemical structure were present in the acetonitrile extract. It is important to note that data presented in Figure 5.8

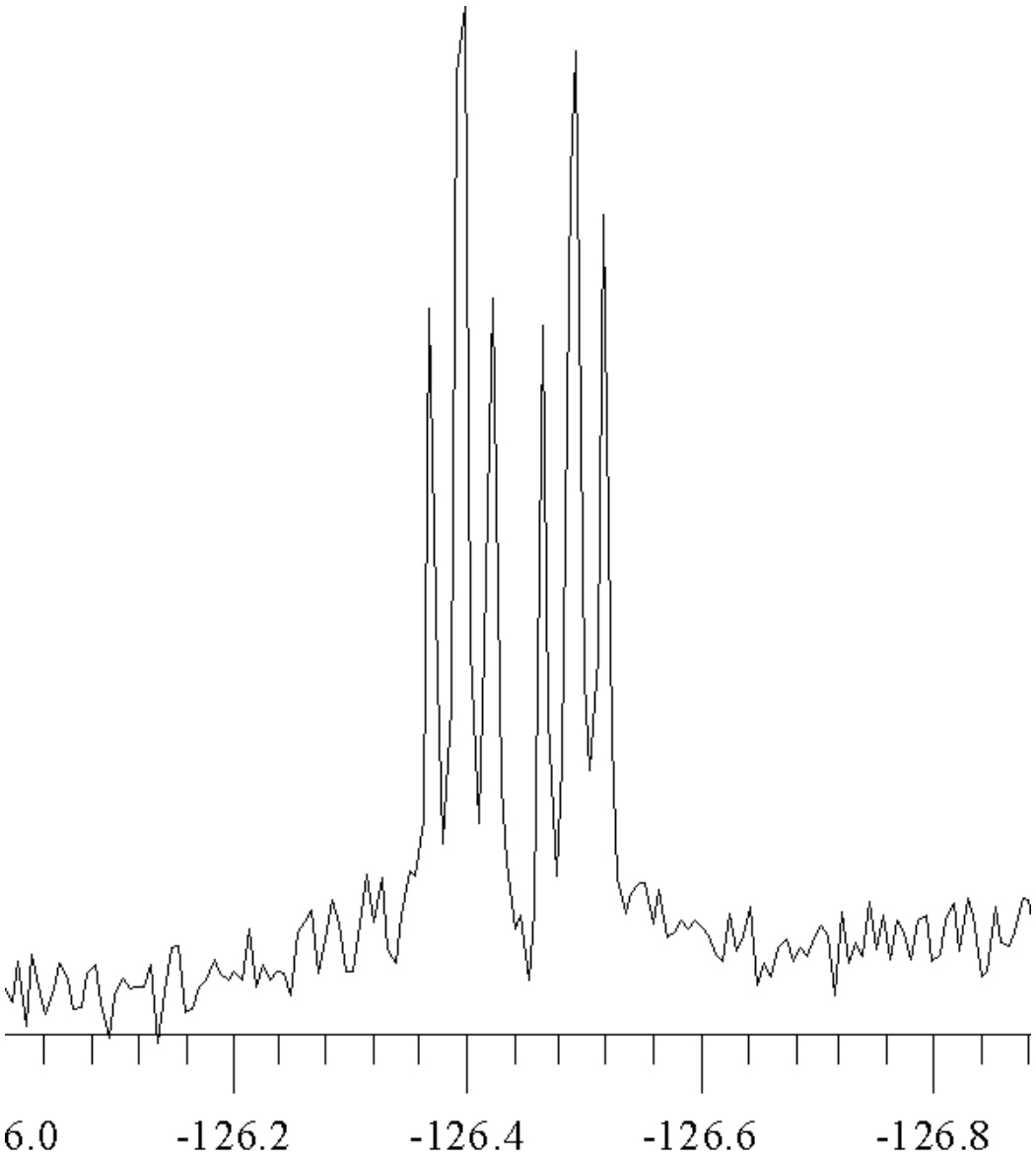


Figure 5.10. A portion of an example spectrum showing the detailed peak splitting of 4-Cl-2-FP metabolites.

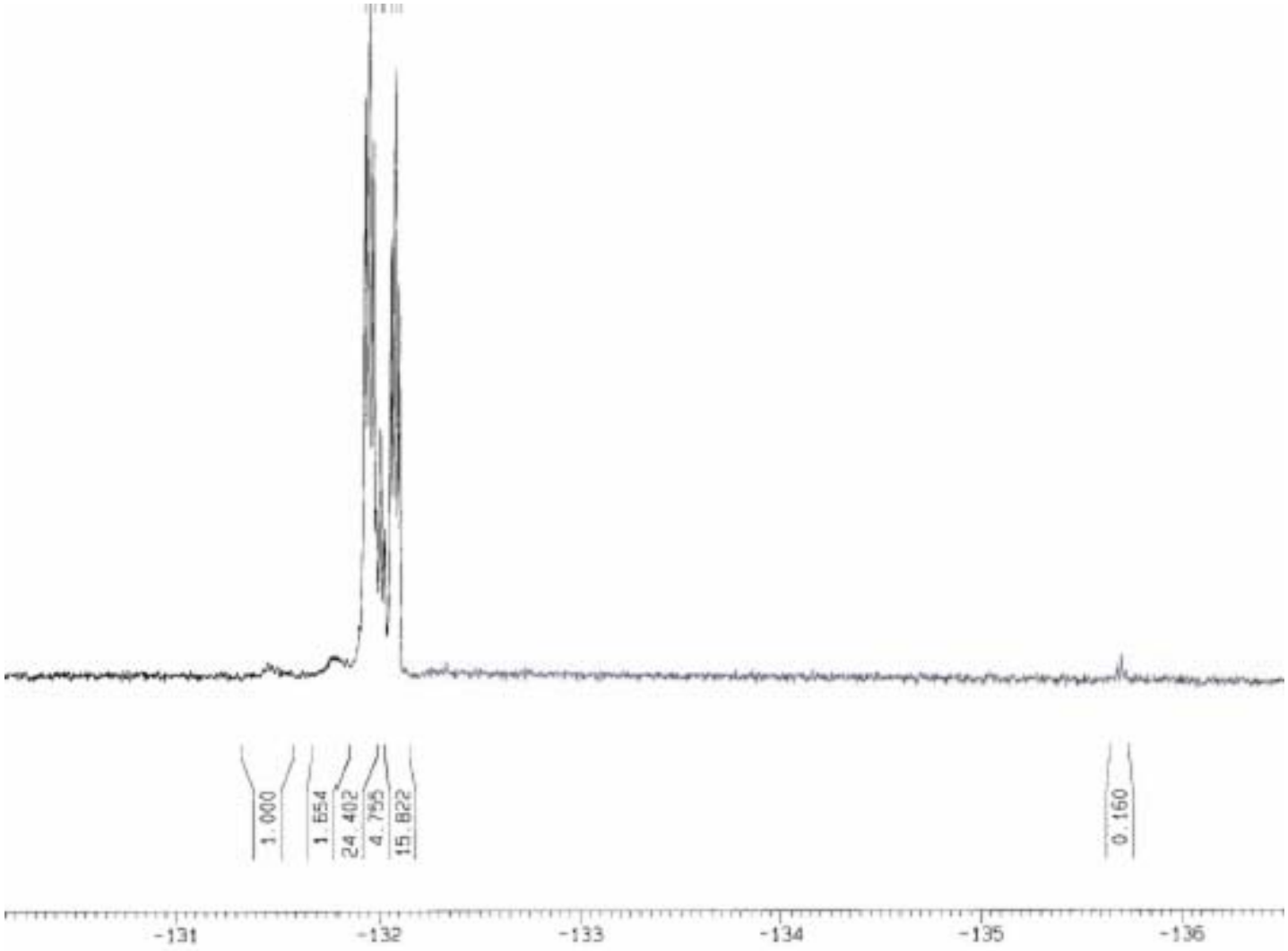


Figure 5.11. Example of spectra gathered with a 500 MHz instrument with a 11.7 T magnet. 4-Chloro-2-fluorophenol (-135.7 ppm) and multiple metabolites (-131.4 to -132.1 ppm) are present.

and Figure 5.11 represent spectra gathered on two separate instruments whose scales were referenced at different times to different samples. The variation in total chemical shift in metabolites observed between metabolites in Figure 5.8 (-126.0 to -126.5 ppm) and metabolites in Figure 5.11 (-131.4 to -132.1 ppm) was attributable to using two instruments for gathering data. The identical shift between metabolites and parent compound demonstrated that the peaks shown in both spectra represented the same parent material and metabolites. All other data for acetonitrile extracts of exposed plants presented herein were gathered on the 400 MHz instrument because the objective of this study was quantification of relative concentrations of parent material and total metabolites and delineating specific differences between metabolites was considered to be a secondary concern of this study.

Peak splitting in parent compounds and metabolites was examined to provide additional information regarding the chemical structure of the metabolites. Peak splitting in ^{19}F NMR is attributed to fluorine – fluorine (F-F) interactions and fluorine - hydrogen (F-H) interactions (Gordon and Ford, 1972). Because all contaminants in this study contained only one fluorine substituent, only F-H interactions were relevant. F-H bonds decrease with increasing number of bonds separating the nuclei and are strongly dependant on chemical geometry (Gordon and Ford, 1972). Peak splitting values for aromatic ring interactions in aqueous samples are 6.2 – 10.1 Hz for a hydrogen ortho to a fluorine; 6.2 – 8.3 Hz for a hydrogen meta to a fluorine; and 2.1 – 2.3 Hz for a hydrogen para to a fluorine (Gordon and Ford, 1972).

Peak splitting values observed for 4-Cl-2FP in acetonitrile extracts were in the range for aromatic ring interactions reported by Gordon and Ford (1972) and example peak splitting values are provided on Figure 5.12. The 9.1 Hz split in the peak representing the downfield metabolite (left) in Figure 5.12 was attributed to the hydrogen positioned ortho to the fluorine (i.e., located the 3-position relative to the hydroxyl substituent). Data provided by Gordon and Ford (1972) indicated that a meta positioned hydrogen would exert weaker influence on peak splitting than the ortho positioned hydrogen. Therefore, upon examination of the upfield metabolite (right) in Figure 5.11b, the 3.4 Hz split in the peak representing the upfield metabolite was attributed to the hydrogen positioned meta to the fluorine (i.e., located in the 6-poisiton relative to the hydroxyl substituent) and the larger split was attributed to the ortho hydrogen. Additional small coupling effects (<3 Hz) were observed infrequently and were attributed to hydrogen positioned para to the fluorine.

Identical peak splitting patterns occurred in the peaks representing parent material and metabolites and it was apparent that hydrogen substituents were in identical locations in parent material and metabolites. Additional peak splitting would indicate metabolites would have experienced an introduction of an additional hydrogen substituent through dechlorination or loss of the hydroxyl moiety. If peak splitting in metabolites was less in number than peak splitting in parent material, metabolites would have been assumed to contain a decreased number of hydrogen substituents due to addition of any type of substituent other than a hydrogen. The identical splitting pattern that occurred between parent material and metabolites indicated that the metabolites detected contained an

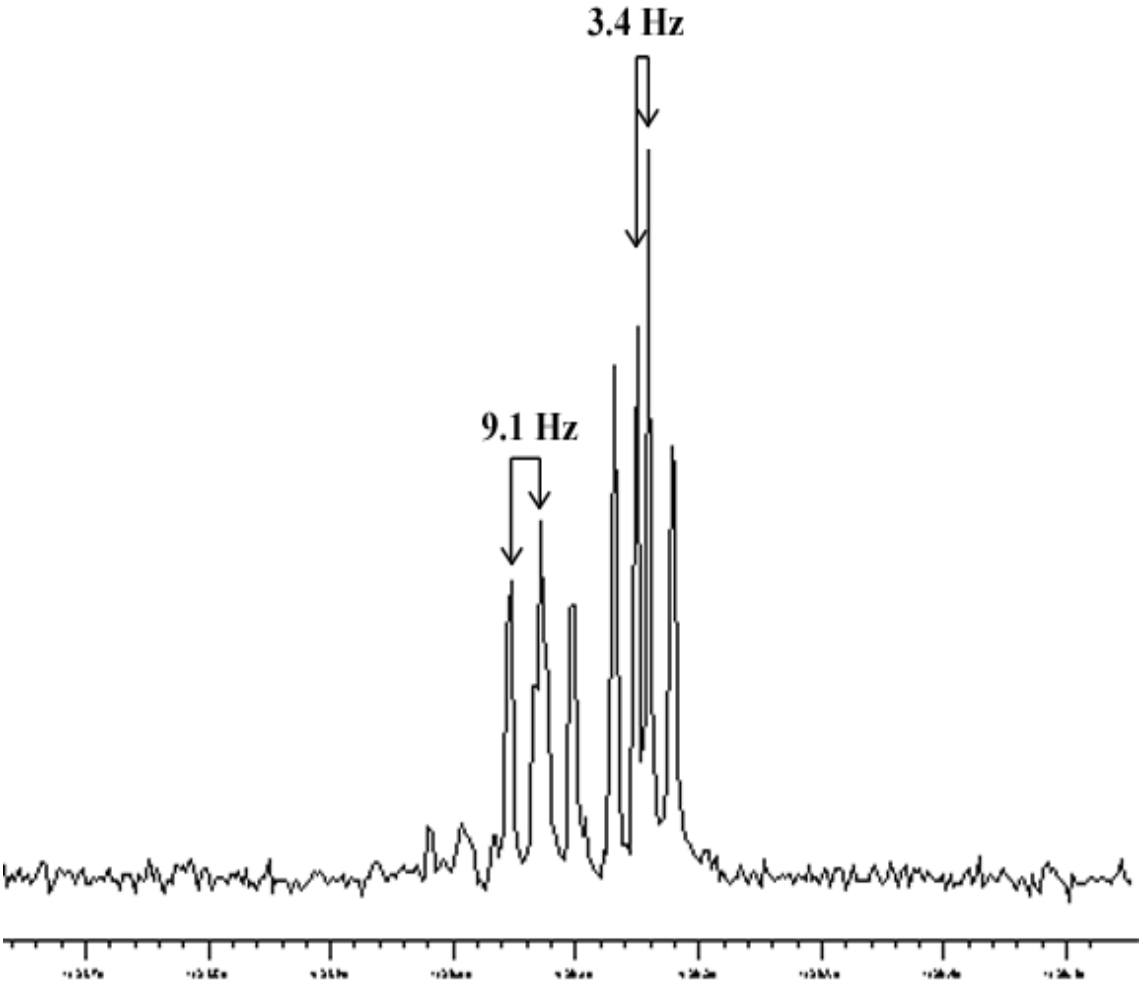


Figure 5.12. Example peak splitting values reported for metabolic products of 4-Cl-2-FP.

identical structure to parent material in terms of halogen and hydrogen substituents. Examination of peak-splitting patterns further confirmed that metabolites detected were conjugated phenols with no modifications to the aromatic ring.

A summary of all peaks identified is presented in Figure 5.13. All peaks in the wide-spectrum, numerous scan spectrum are labeled as parent material, conjugated metabolites or spectrum artifacts. ^{19}F NMR results were consistent with previous reports by Day (2002) that conjugation of the hydroxyl moiety of halogenated phenols provided the primary metabolic products produced by plants.

Quantification of Chlorophenols in Liquid Extracts via ^{19}F NMR

Batch experiments where plants were exposed to various concentrations of 4-Cl-2-FP for various lengths of time were used to obtain kinetic information regarding production of 4-Cl-2-FP metabolites. Experiments were conducted with relatively low concentrations of 4-Cl-2-FP where oxygen production measurements verified that systems were uninhibited. Extracts of plant materials were analyzed with a narrow spectral window centered on the area of interest (40 ppm) and the number of scans necessary to provide spectra containing quantifiable peaks.

Quantification of 4-Cl-2-FP and its metabolites internal to plants was achieved using plant tissue extracts. To quantify parent material and metabolites in samples, areas of peaks of interest (parent material and metabolites) were compared with areas of peaks of

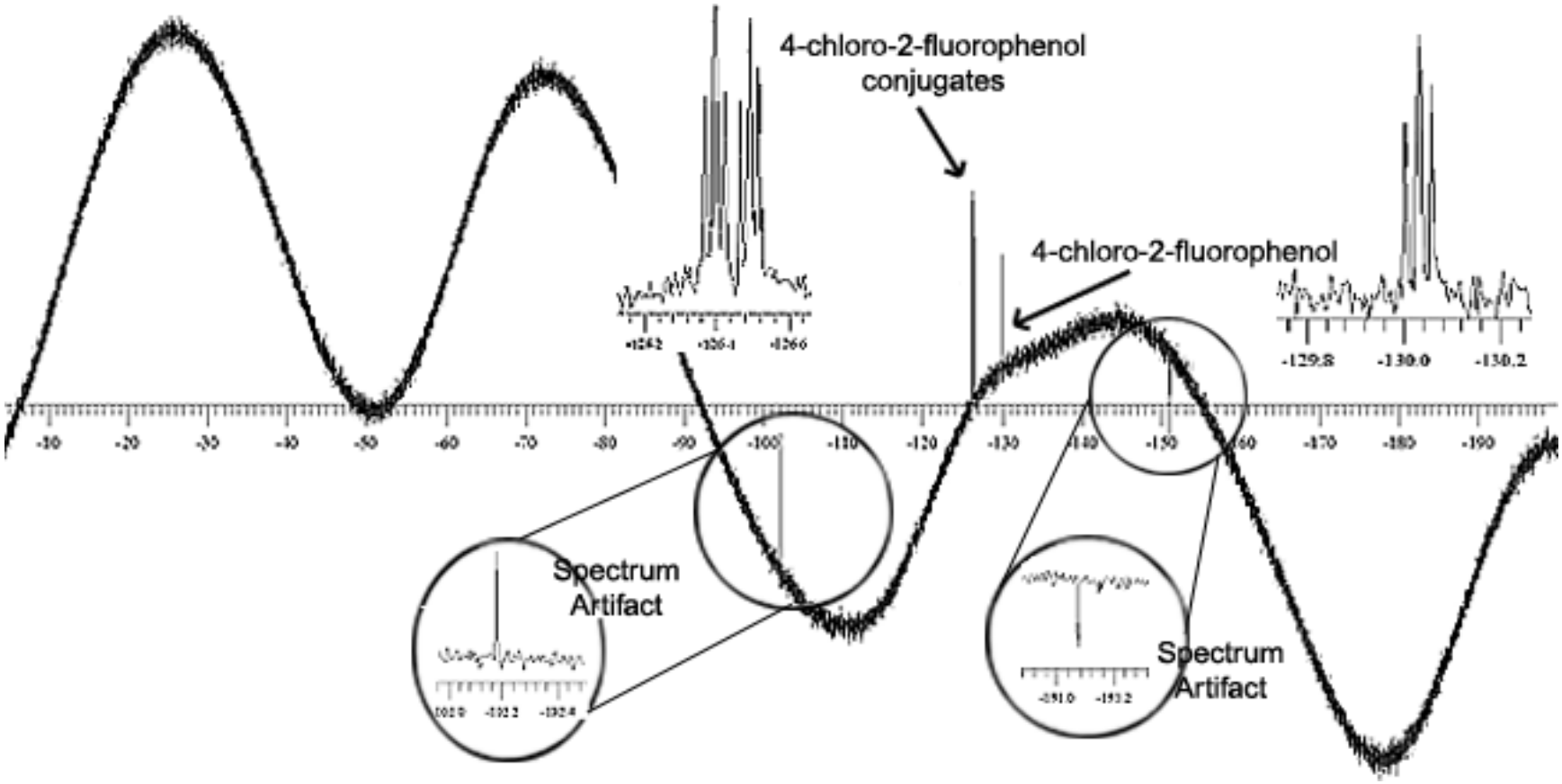


Figure 5.13. Example of spectra gathered with a large spectral window and 20,000 scans. 4-Chloro-2-fluorophenol (-130.1 ppm) and metabolites (-126.0 to -126.5 ppm) were present along with two spectral artifacts (-102.3 and -151.0)

an internal standard which possessed a chemical shift in the same range as the compounds of interest. Fluorobenzene and 2,6-chloro-4-fluorophenol were used as internal standards with chemical shifts of -109.7 ppm and -118.2 ppm, respectively. Example spectra are shown in Figures 5.14 and 5.15. A known mass of a known concentration of an internal standard (dissolved in deuterated acetonitrile) was added directly to the NMR tube. Sample length in the NMR tube was recorded and used to calculate the total volume of sample, based on manufacturer information regarding diameter of NMR tubes. Peaks were integrated electronically after baseline phasing and baseline correction. Using the knowledge that the peak areas are proportional to the number of nuclei present, molar amounts of 4-Cl-2-FP and 4-Cl-2-FP metabolites were calculated by comparison of peak area between the internal standard peak and the 4-Cl-2-FP and 4-Cl-2-FP metabolite peaks.

A comparison of spectra representing extracts of plants exposed to 4-chloro-2-fluorophenol for 12 and 48 hr, and an unexposed extract spiked with the parent material, is shown in Figure 5.16. These results show the presence of the parent compound existing in plant extracts at 12 and 48 hr, identified by the resonance line at -130.0 ppm. Fluorinated metabolites were also present, as was demonstrated by resonance lines in the range of -126.0 to -126.5 ppm. Peaks for metabolites at 48 hr are larger than those at 12 hr, indicating larger molar quantities of metabolites at 48 hr than 12 hr. These results indicated that a greater quantity of metabolites accumulated internal to plants after longer aqueous exposure.

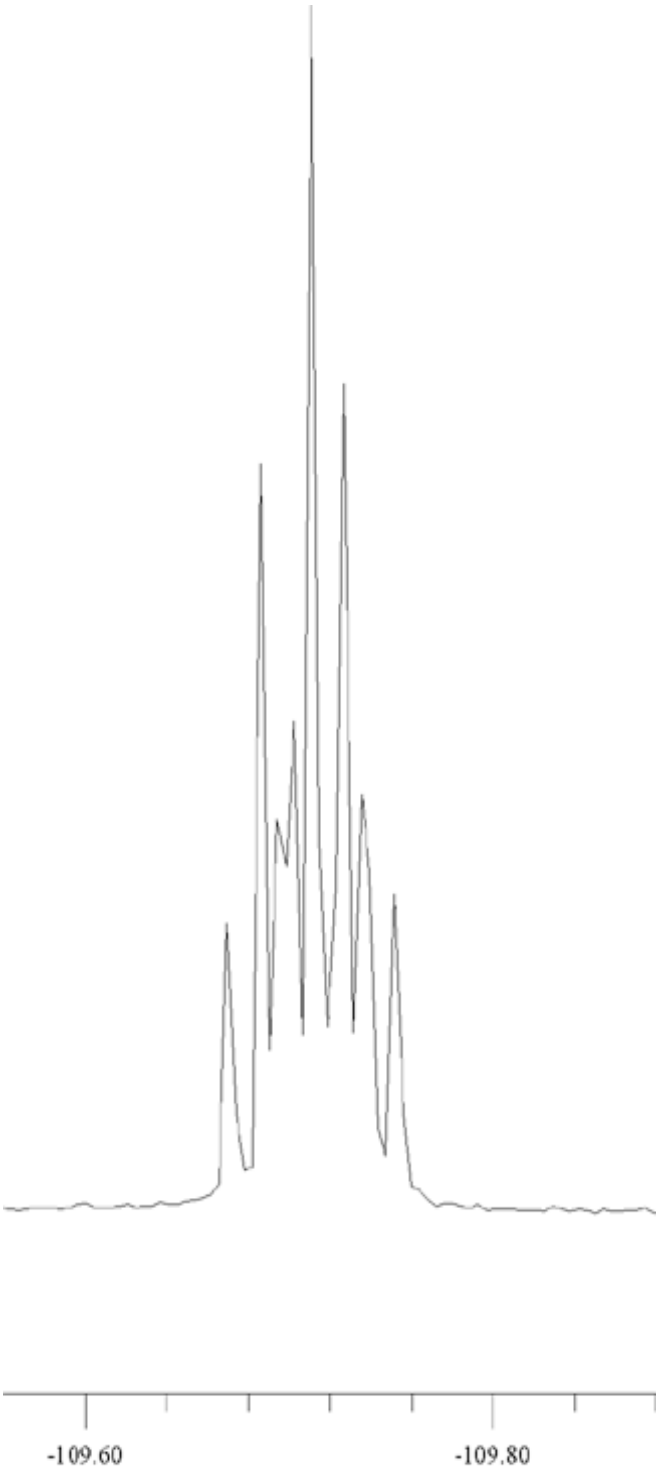


Figure 5.14. Example spectrum of an internal standard fluorobenzene.

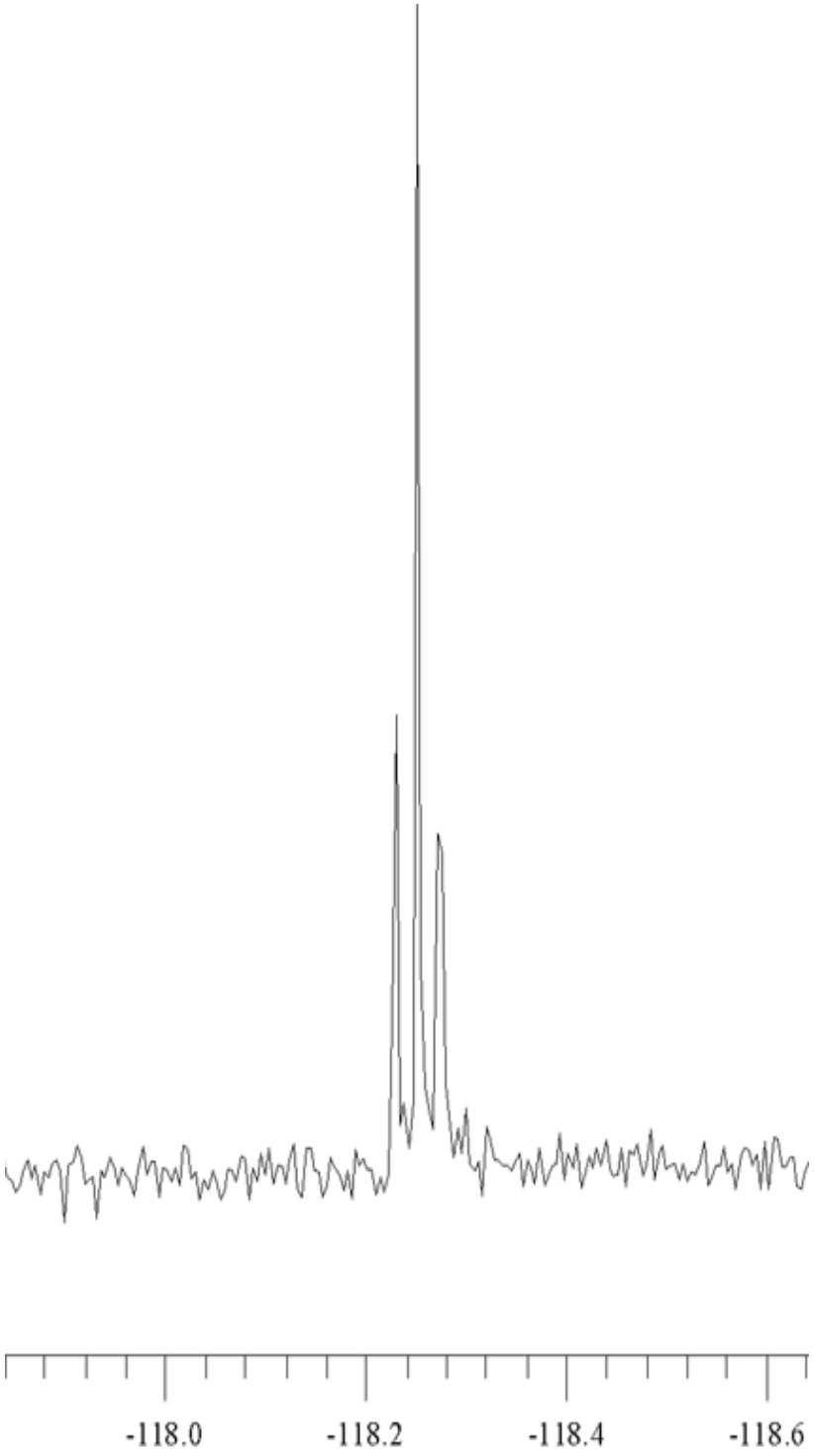


Figure 5.15. Example spectrum of an internal standard 2,6-chloro-4-fluorophenol.

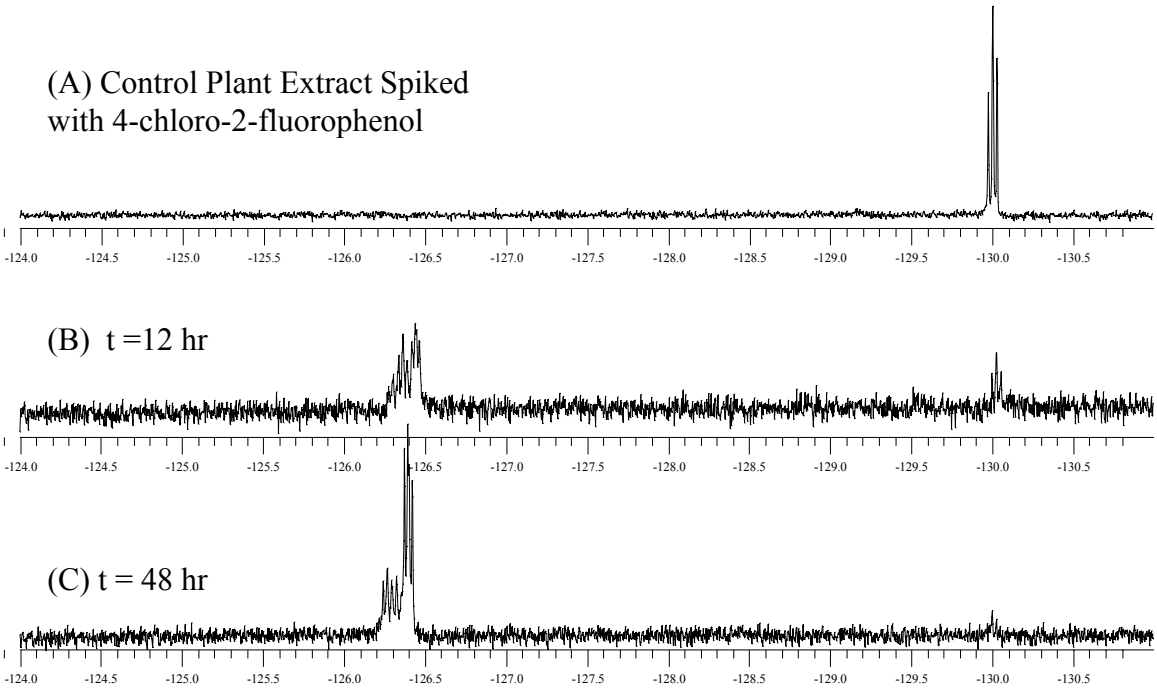


Figure 5.16. ^{19}F NMR data comparing a control plant extract spiked with the 4-chloro-2-fluorophenol (A) with extracts of plants exposed to 4-chloro-2-fluorophenol for 12 and 48 hr (B and C). Metabolites are seen in the chemical shift range from -126.2 to -126.5 ppm whereas the parent compound is observed at -130.0 ppm.

Data gathered from an experiment with an initial aqueous phase concentration of 10 mg 4-Cl-2-FP/L, plant mass of 4.5 g, a pH value of 7, and an aqueous volume of 200 mL are shown in Figure 5.17. 4-Cl-2-FP was continuously removed from the aqueous phase and the parent material was detected in plant extracts at 15, 39 and 48 hr. Concentrations of 4-Cl-2-FP detected in plant tissue ranged from 0.058 to 0.127 μmol/g plant, which represented < 5% of the total mass in the reactor (2.94 μmol/g plant). Metabolites of 4-Cl-2-FP were also detected in plant tissue and were seen to increase from 0.279 μmol/g at 15 hr to >1 μmol/g at 48 hr. Metabolites represented a small fraction of the total mass of 4-Cl-2-FP in the reactor at 15 hr (10%) and increased at 48 hr (36%). Mass balance data are given as a percentage over the aqueous phase data for each time point in Figure 5.17. Total mass recovered ranged from 76 – 90% with decreasing mass recoveries at later time points.

Data gathered from an experiment with an initial aqueous-phase concentration of 19 mg 4-Cl-2-FP/L, a pH value of 7, plant mass of 4.5 g and an aqueous volume of 200 mL are shown in Figure 5.18. 4-Cl-2-FP was continuously removed from the aqueous phase and the parent material was detected in plant extracts at level of 0.126 μmol/g, 0.107 μmol/g and 0.0307 μmol/g at 10, 24 and 77 hr, respectively. Concentrations of internal 4-Cl-2-FP detected in plant tissue represented <2% of the total mass in the reactor (6.56 μmol/g plant). Metabolites of 4-Cl-2-FP were also detected in plant tissue and were seen to increase from 1.238 μmol/g at 10 hr to 3.508 μmol/g at 77 hr. Metabolites represented an increasing fraction of the total mass of contaminant in the reactor, comprising only

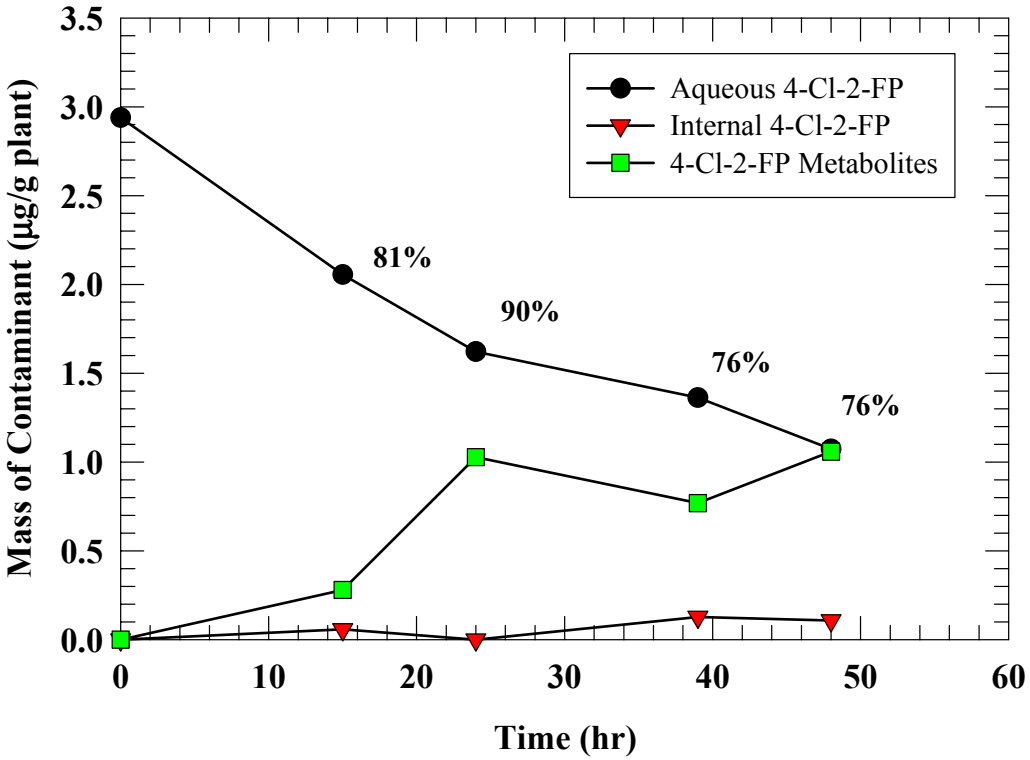


Figure 5.17. 4-Chloro-2-fluorophenol uptake by *L. minor* with internal parent material and metabolic products measured using ^{19}F NMR analysis of plant extracts. Mass balance closure information is given as a percentage over the aqueous phase data for each time point.

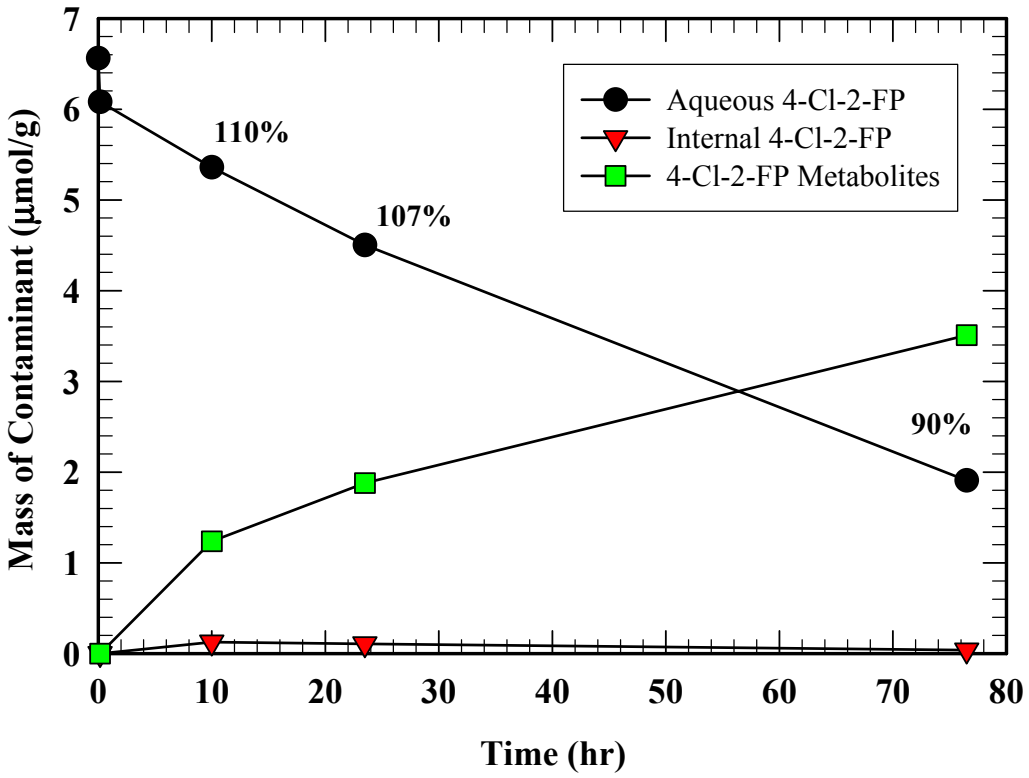


Figure 5.18. 4-Chloro-2-fluorophenol uptake by *L. minor* with internal parent material and metabolic products measured using ^{19}F NMR analysis of plant extracts. Mass balance closure information is given as a percentage over the aqueous phase data for each time point.

18.9% of contaminant in reactor at 10 hr and 28.6% at 24 hr reaching 53.5%, at 77 hr. Mass balance closure information is given as a percentage over the aqueous phase data for each time point. Total mass recovered ranged from 90 – 110% with decreasing mass recoveries at later time points.

Discussion

Contaminant fate in aquatic plant systems was examined using ^{14}C-labeled chlorophenols. ^{14}C-2,4,5-TCP and ^{14}C-2,4-DCP were used as a tracer in the plant reactor systems to determine the fate of chlorophenols removed from the aqueous phase. ^{14}C-labeled compounds were removed from aqueous phase measured via HPLC in parallel with removal of 2,4,5-TCP and 2,4-DCP from aqueous phase with no net release into the aqueous phase. Oxidation of plant material after 2,4,5-TCP uptake indicated that ^{14}C-2,4,5-TCP removed from the aqueous phase was sequestered in the plant material. Results showed that plants accumulated 73 (±2)% of 2,4,5-TCP and 94 (±5)% of 2,4-DCP in reactors by 48 hr, indicating that the majority of contaminant in reactors was in the plant phase. No significant accumulation of ^{14}C material was observed in reactor headspace and quantities observed in headspace were expected given the Henry's Law constants in the range of 4.33×10^{-3} atm·L^{3}·mol^{-1} (Howard and Meylan, 1997). Results demonstrated that 2,4,5-TCP depletion from aqueous phase was a result of net contaminant movement into plant tissue and indicated that *L. minor* rapidly sequestered 2,4,5-TCP internal to the plant with no net contaminant release into aqueous phase by plants over a 96-hr experimental time frame.

Sequestration of contaminants and production of metabolites by aquatic plants was examined using fluorinated analogs of chlorophenols and ^{19}F NMR. Analysis of liquid extracts of plants exposed to 4-Cl-2-FP using ^{19}F NMR revealed that low concentrations of parent material remained internal to plants. In addition, resonance lines detected downfield of parent material represented metabolic products of 4-Cl-2-FP sequestered internal to plants. Metabolic products were similar to parent material in chemical structure, with small chemical shift and peak splitting patterns indicating that metabolites were conjugated at the hydroxyl position. Quantification of 4-Cl-2-FP and its metabolites internal to plants showed that parent material comprised a relatively small quantity (< 10%) of contaminant internal to plants in uninhibited systems. In uninhibited systems, the majority of plant sequestered material was in the form of extractable metabolic products.

The pattern of plant accumulation and sequestration of chlorinated phenols demonstrated by ^{14}C and ^{19}F NMR data was consistent with data presented by Day (2002) and Day and Saunders (2004) which definitively tracked plant processing of chlorophenols into vacuoles and cell components. Results presented herein achieved ~100% material recoveries thus enhancing the data of Day (2002) and Day and Saunders (2004) where methods used to identify and track contaminants as they moved into increasingly complex molecules internal to plants. Upon complete removal of chlorophenols from the aqueous phase (90 – 144 hr), Day (2002) identified 79(±3)% of 2,4,5-TCP and 50(±5)% of 2,4-dichlorophenol as glycoside derivatives or parent compound internal to plants, with the remaining portion represented by chlorophenol incorporated into cell wall tissue

or vacuole material. The ^{14}C data indicated definitively that 2,4,5-TCP and 2,4-DCP removed from the aqueous phase were sequestered within plants. In addition, ^{19}F NMR data demonstrated that measurable quantities of parent chlorophenols existed internal to plants and that the majority of chlorinated phenol was in the form of extractable metabolite internal to plants.

CHAPTER 6

ROLE OF PLANT ACTIVITY AND CONTAMINANT SPECIATION IN AQUATIC PLANT ASSIMILATION OF ORGANIC CONTAMINANTS

There is a lack of critical understanding of how contaminant exposure or contaminant transformation affects plant metabolic activity. To develop a system in which contaminant uptake is a sustainable and continuing process, as required in natural and engineered phytoremediation systems, an enhanced understanding of contaminant speciation and the dynamic processes that affect plant-contaminant interactions is necessary. Interactions between plant metabolic activity and uptake of an ionizable organic contaminant were explored. Contaminant uptake, plant metabolic activity and contaminant speciation were integrated to obtain an advanced understanding of processes responsible for plant-contaminant interactions. Rapid uptake and sequestration of 2,4,5-TCP by *L. minor* were established in previous chapters, therefore efforts were focused on *L.* and 2,4,5-TCP.

Assessment of Plant Activity (α)

A reliable method for determining plant metabolic activity was necessary to gauge effects of background plant metabolic activity on contaminant uptake rate. In addition, plant activity measurements were used to assess inhibition of plant systems by contaminants or external factors. All data presented in Chapter 6 represent plants which were not

inhibited as per inspection of plant activity data. Results gathered for inhibited systems are presented in subsequent sections.

Oxygen Production Rate (α) as a Measure of Plant Activity.

Oxygen production rate has been used to measure photosynthetic activity for use as an indicator of plant metabolic activity (*e.g.,* Fleming et al., 1995; Kemp et al., 1986). Results from a representative experiment conducted without contaminant addition where headspace analyses were performed on triplicate reactors over a 24-hr period revealed a constant and continuous oxygen production rate of 14.4 (±1.0) μmol/hr per gram plant (Figure 6.1). The constant oxygen production rate in contaminant-free plant systems allowed for its use as an indicator of plant metabolic activity, provided it was assessed for each batch of *L. minor* used experimentally. Oxygen production rate data gathered for contaminant-free systems indicated the repeatability and reliability of such measurements. Use of oxygen production rate facilitated comparison between experiments and normalization of plant activity. An illustrative example of oxygen production rate measurements for reactors which were sacrificed after 0, 24 and 48 hr of exposure is shown in Figure 6.2. Data presented represent triplicate reactors of control plants which underwent the same treatment as TCP-treated plants, without contaminant exposure. Oxygen production rate was continuous over 48 hr for plant exposed in media fixed at pH values of 6 and 8 as was demonstrated by the fact that no statistical difference was observed between oxygen production rate values for plant sacrificed after 0, 24 and 48 hr of exposure in the same experiment. For example, plant activity measurements for controls plants in the pH 8 system shown in Figure 6.2 were 6.3±1.3, 4.9±0.8, and

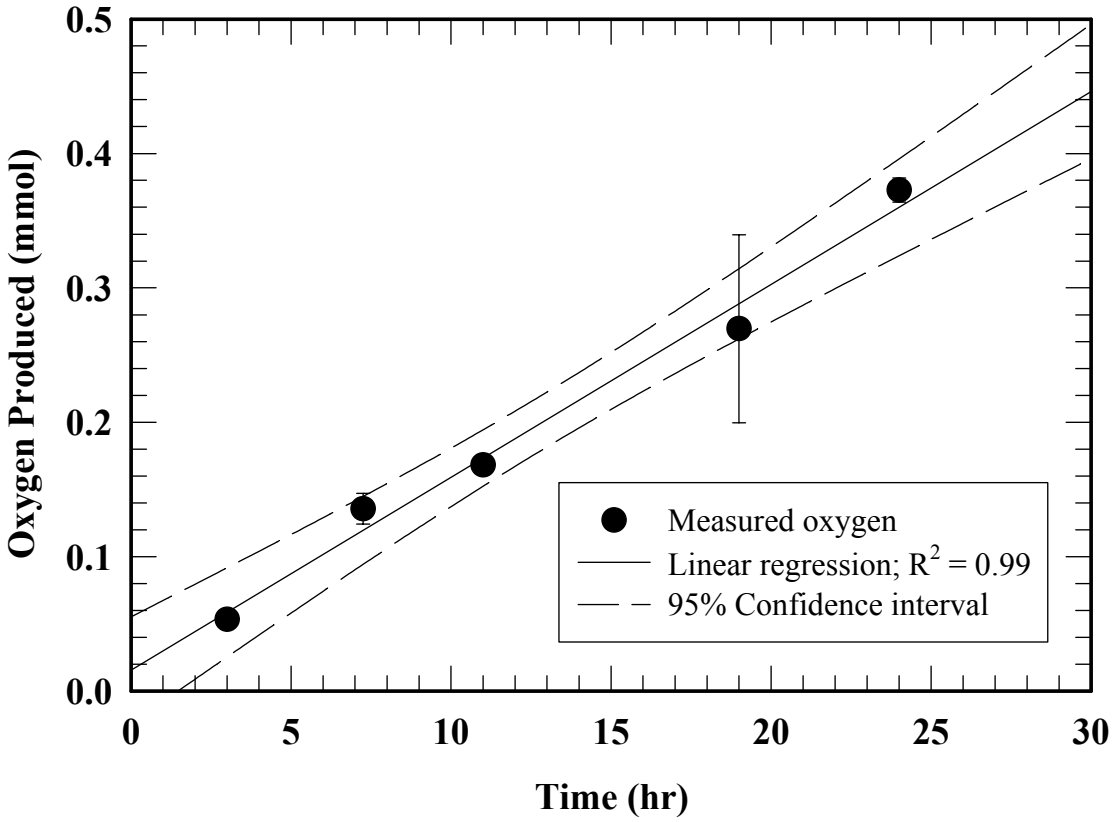


Figure 6.1. Oxygen produced with time for *L. minor* in uninhibited control plant systems indicating a constant oxygen production rate (α) of 14.4 ± 1.0 μmol/hr for 1 g fresh weight of plant.

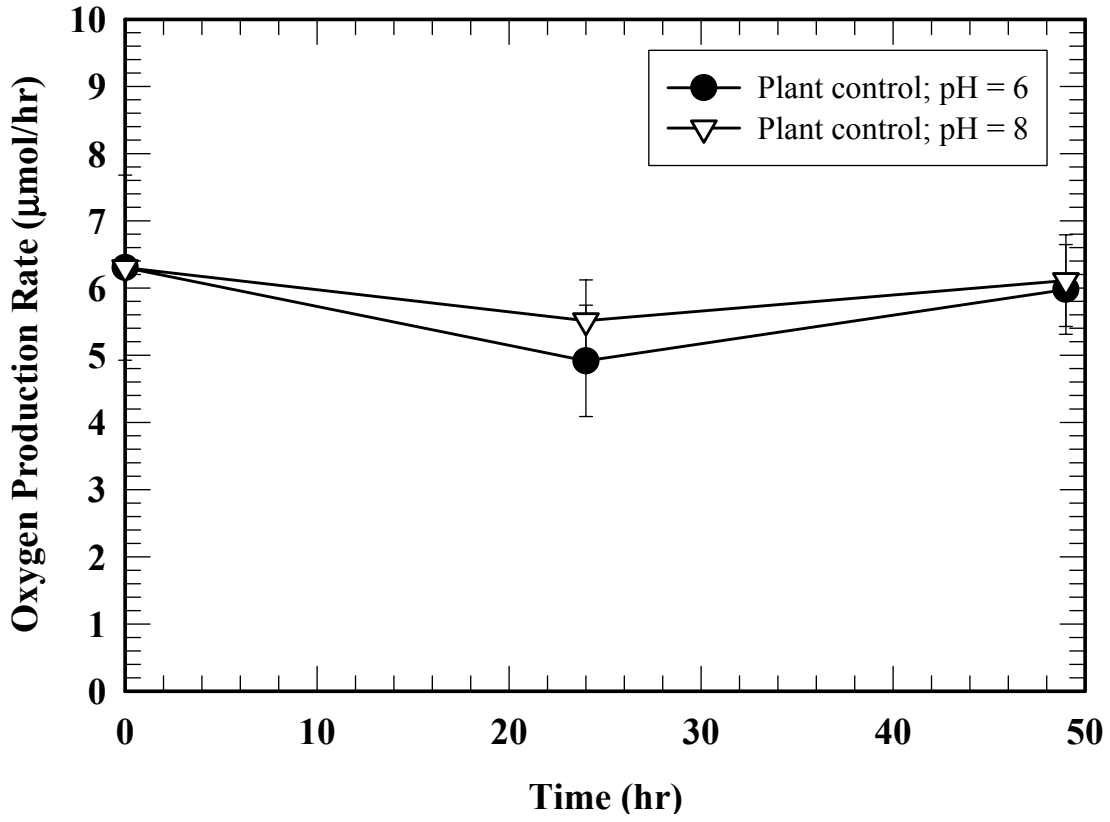


Figure 6.2. Illustrative oxygen production rate measurements for non-contaminated control systems exposed at pH values of 6 and 8. Oxygen production rate was continuous through the time course of the experiment.

5.9±0.7 μmol/hr for 0, 24 and 48 hr, respectively demonstrating that no statistical difference existed for plant activity in control plant systems and that plant metabolic activity was constant with time in control systems. These results further demonstrated that control plant activity did not depend on media pH. Results demonstrating that oxygen production rate was constant with time in control systems confirmed the use of oxygen production rates as an indicator of plant activity and health in similar systems.

The parameter, α, was used to represent plant metabolic activity and a range of α values (i.e., α = 3.4 – 34.7 μmol/hr) was measured in numerous batch experiments conducted over a period of several years. The relative standard deviation for α values ranged from 4.4 – 22%, with values typically ~10%. Plant activity data were gathered over the course of several years and plant activity values varied during that time. Plant activity values are compared with date of measurement in Figure 6.3. These data show the 10-fold range of plant activity values observed and demonstrate that there was not a strong time bias underlying plant activity measurements. The distribution of α values observed is presented in Figure 6.4, where frequency of observation is compared with value observed. This distribution of α values demonstrated that observations of high plant activity occurred less frequently than observations of low plant activity; however high plant activity values were observed with great enough frequency (i.e., $\alpha > 0.025$ mmol/hr was observed 12 times) that these data were not considered to be erroneous. The range of α values observed was not typical for an individual batch experiment since the values with individual triplicate experiments varied by ~10% of the mean value. The 10-fold range of α values along with relative standard deviation values of ~10% demonstrated the

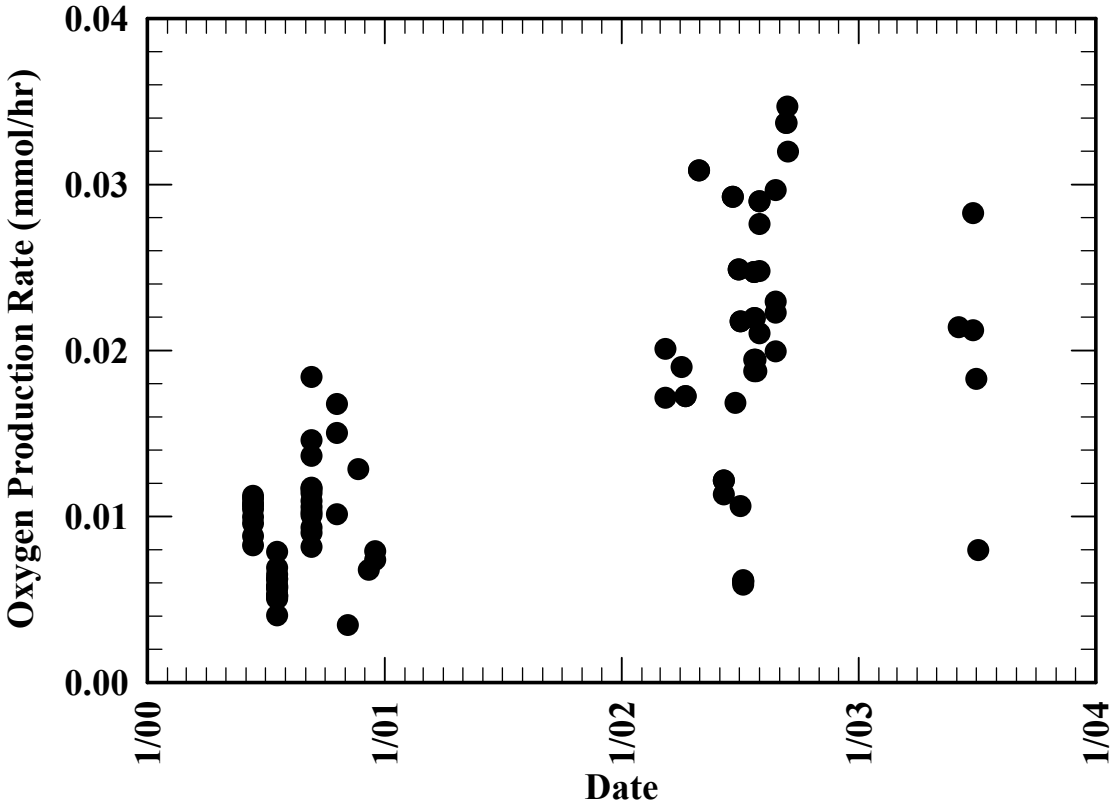


Figure 6.3. Plant activity data are compared with date on which data were gathered.

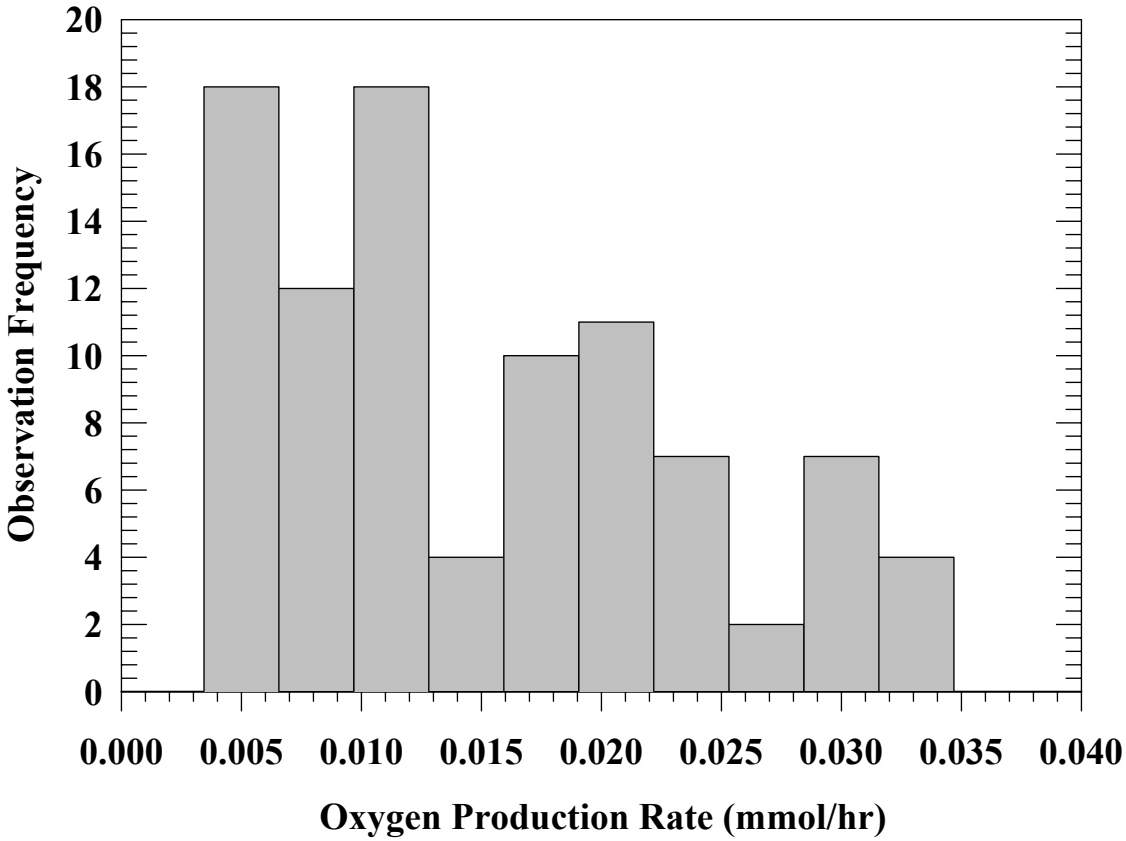


Figure 6.4. The histogram for oxygen production rate values contains bin widths of 0.00313 mmol/hr and values were distributed between 0.00344 and 0.0347 mmol/hr.

high variability that exists in aquatic plant systems and demonstrated that plant metabolic activity was an important variable.

Integration of Plant Activity, Contaminant Speciation and Contaminant Uptake Rate.

An analysis of relationships between plant activity and contaminant uptake rate was performed using oxygen production rate to gauge plant activity in experimental systems. The aquatic plant *L. minor* was the model plant and 2,4,5-TCP was used as the model contaminant. Contaminant speciation was examined as an additional factor in contaminant uptake rate.

Plant Activity Effects on Contaminant Uptake and Assimilation.

Batch experiments were used to examine links between plant metabolic activity (α) and contaminant uptake, where α was assessed as oxygen production rate of control systems as previously defined in Chapter 3. Plant activity measurements taken in conjunction with aqueous 2,4,5-TCP measurements demonstrated that uptake of 2,4,5-TCP from the aqueous phase was a first-order process when oxygen production rates were unaffected by contaminant exposure, i.e., when α values for 2,4,5-TCP exposed system were not statistically different from α values in control systems. Supporting data for this concept are shown in Figures 6.5 – 6.7, where oxygen production measurements were taken simultaneous to aqueous 2,4,5-TCP measurements. At an initial 2,4,5-TCP concentration of 2.1 mg/L in Figure 6.5, 2,4,5-TCP removal proceeded in an exponential manner for plants with media pH of values of 6 and 8, with removal rate being higher at a pH value

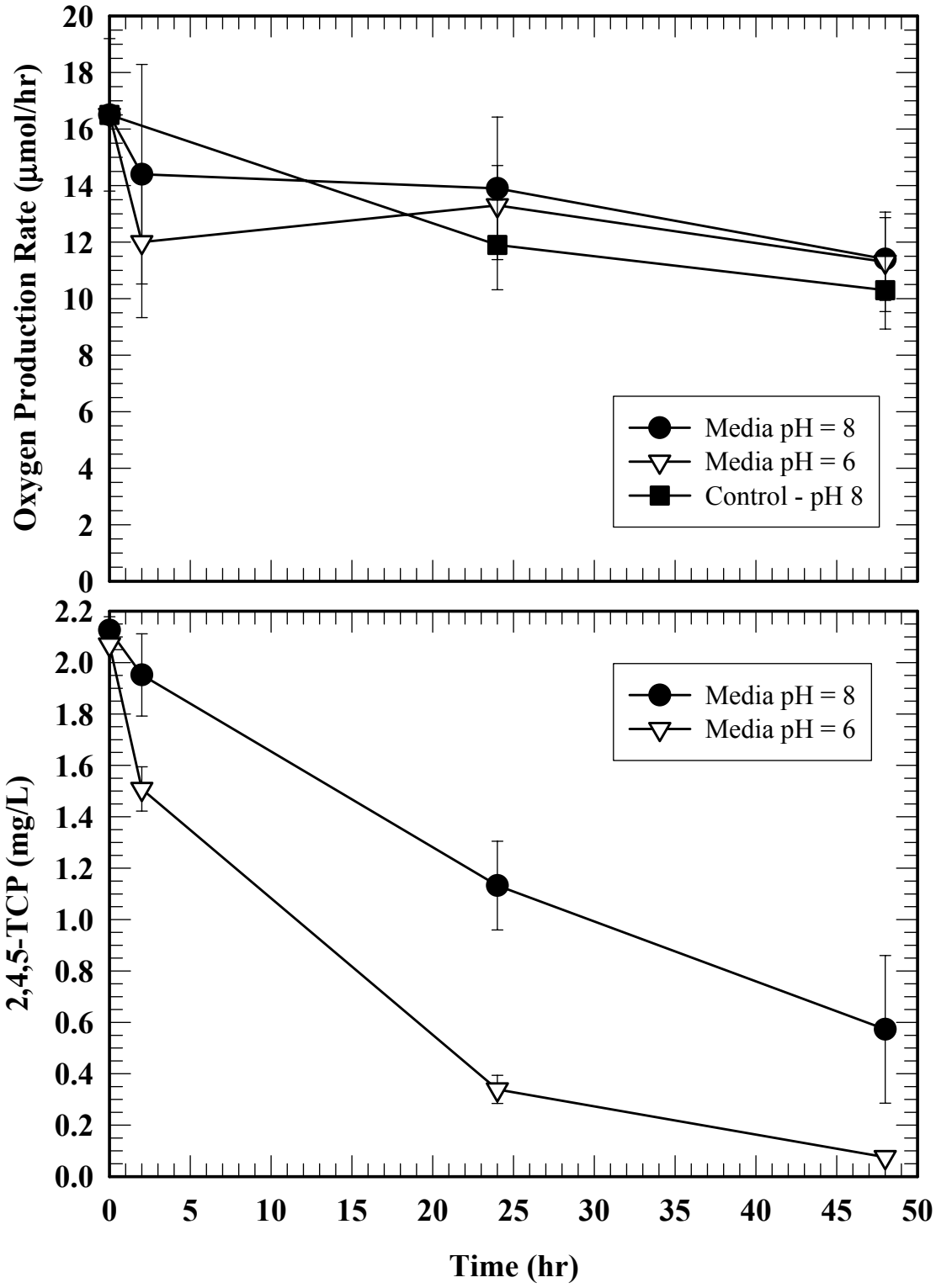


Figure 6.5. Aqueous phase 2,4,5-TCP and oxygen production rate at pH values of 6 and 8.

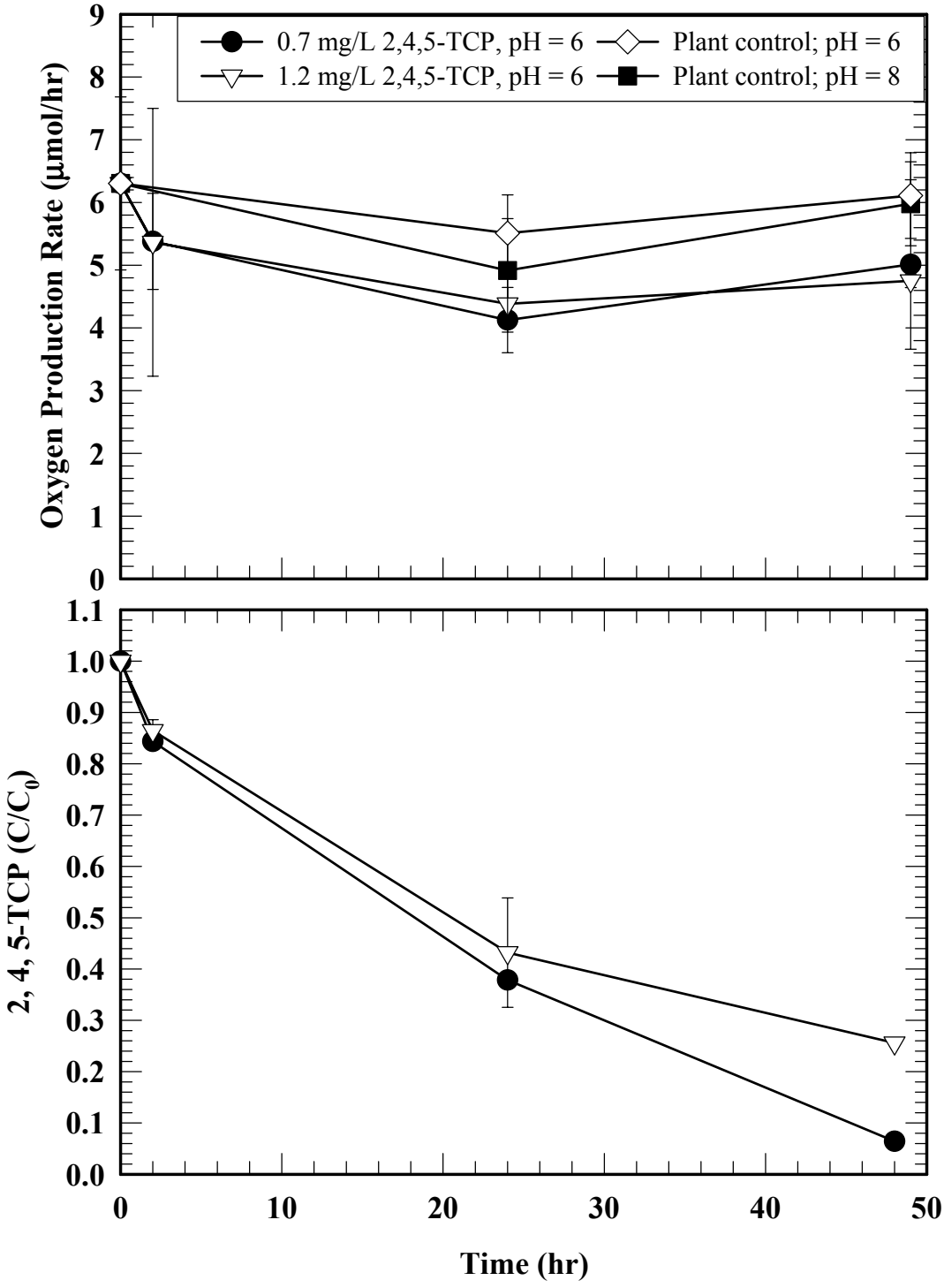


Figure 6.6. Aqueous phase 2,4,5-TCP and oxygen production rate at a pH value of 6.

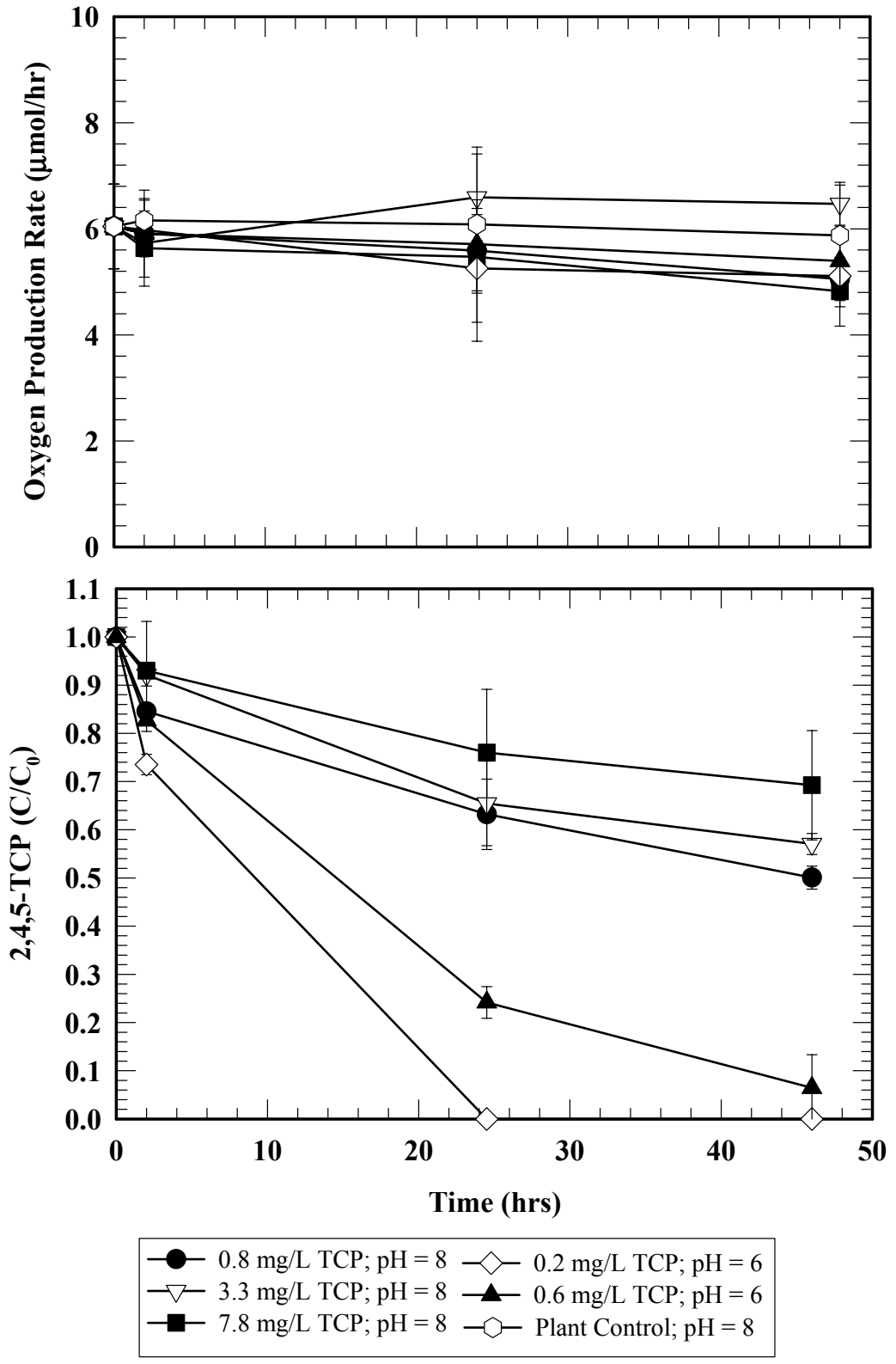


Figure 6.7. Aqueous Phase 2,4,5-TCP and oxygen production rate at pH values of 6 and 8.

of 6. The oxygen production rates in 2,4,5-TCP exposed systems for samples taken at 24 and 48 hr were statistically the same as oxygen production rates of control systems indicating no inhibitory effects were observed. For example, after 48 hr of contaminant exposure, α values for control, pH 6 and pH 8 systems presented in Figure 6.5 were 10.2±1.3, 11.3±1.7, and 11.4±1.5 μmol/hr. Oxygen production rate for the initial time point in control systems (t = 0 hr) was greater than that of samples taken at 24 and 48 hr. The drop in oxygen production rate for control systems presented in Figure 6.5 was not consistently observed in all systems, where oxygen production rate values frequently increased after t = 0 (e.g., Figure 6.7). Therefore, small fluctuations in control plant activity measurements were a product of variability that exists in aquatic plant systems. In Figure 6.6, 2,4,5-TCP concentrations of 0.7 and 1.2 mg/L were examined at a pH value of 6. At both concentrations presented in Figure 6.6, oxygen production indicated no significant inhibition and, likewise, 2,4,5-TCP uptake rate proceeded exponentially. Finally, a similar study with 2,4,5-TCP ranging from 0.2 to 7.8 mg/L and pH values of 6 and 8, indicated that uninhibited systems proceeded to rapidly remove 2,4,5-TCP (Figure 6.7). In Figures 6.5 and 6.7, there were clear indications of pH effects which are addressed in a subsequent section. Therefore, the above data show rapid 2,4,5-TCP uptake occurred in systems where plant oxygen production equaled the control.

First-order rate projections for example systems are shown in Figure 6.8 for selected data from Figures 6.5 – 6.7 which represent a variety of pH values, initial concentrations and α values. Comparison of α values for 2,4,5-TCP-treated experimental systems with

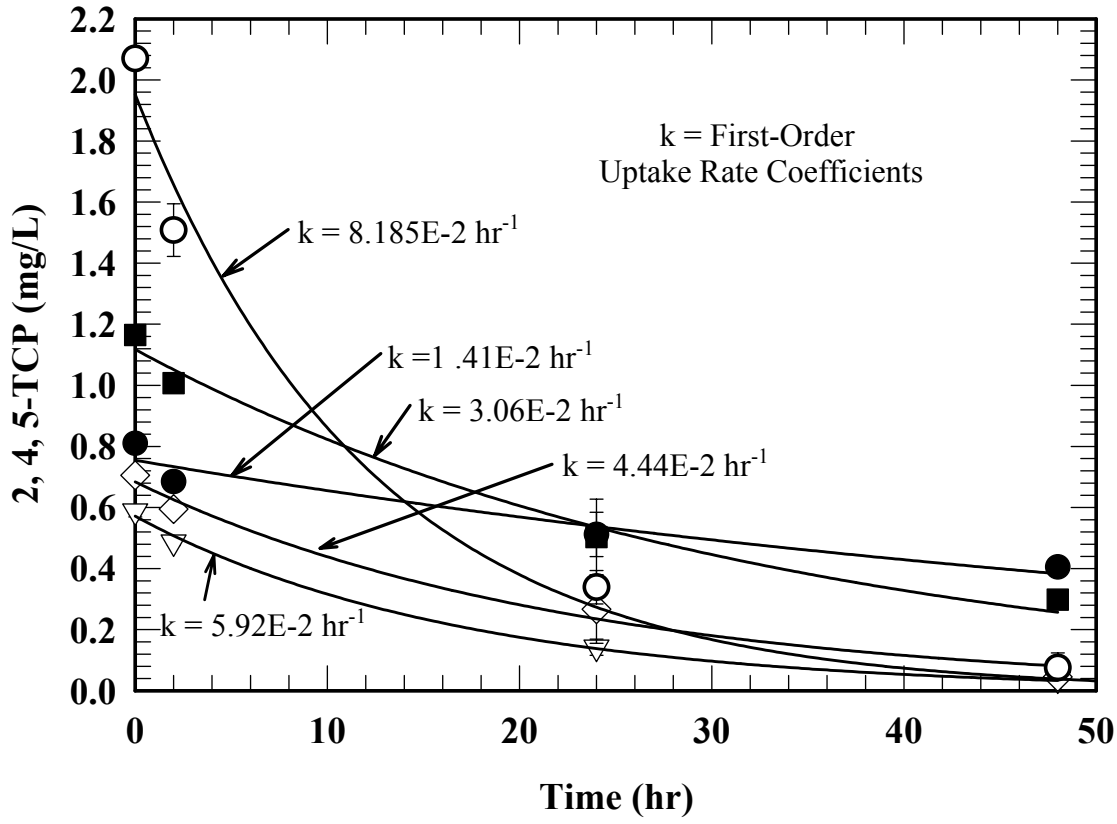


Figure 6.8. Contaminant uptake rates provided for example reactor systems where no plant inhibition was observed. Data shown represent a variety of pH values, initial 2,4,5-TCP concentrations, and plant activities. The first-order rate coefficient, k, found through non-linear regression is indicated for each plot.

α values for live control systems demonstrated that plants were not inhibited by 2,4,5-TCP exposure over the time course of the study. First-order uptake rate coefficients, k, for example data in Figure 6.8 are presented to illustrate that uptake rate did not directly correspond with initial 2,4,5-TCP concentration. These data and other data sets lead to the need for inspection of other system variables, such as media pH and plant activity.

Correlations between α values and contaminant uptake rate coefficient (k) in uninhibited-plant systems are shown in Figures 6.9 and 6.10 for experiments at pH values of 6 and 8, respectively. In these figures, first-order uptake rate coefficients (k) for 2,4,5-TCP removal were assessed for individual experimental systems. To enhance statistical assessments, data points are shown for each experimental reactor instead of averages of replicates where possible (i.e., where reactors were not sacrificed during the time course of the experiment for inhibition assessment). First-order rate coefficients shown in Figures 6.9 – 6.10 were for systems in which α values indicated there was no inhibition of photosynthetic activity during the course of an experiment and all first order rates were determined after sorptive losses were incorporated (see Chapter 4). As described previously, the variations in plant uptake rates were those inherent to plant systems and these data indicated the critical need to independently assess plant activity in phytoremediation of contaminants. To quantify the relationship between plant activity and contaminant uptake rate, linear correlations between plant oxygen production rate, α, and 2,4,5-TCP uptake rate coefficient, k were developed for various pH values examined (Figures 6.9 and 6.10). Each data set is presented with linear regression and 95% confidence intervals on the slope parameter. The y-intercept value for the pH 8 system

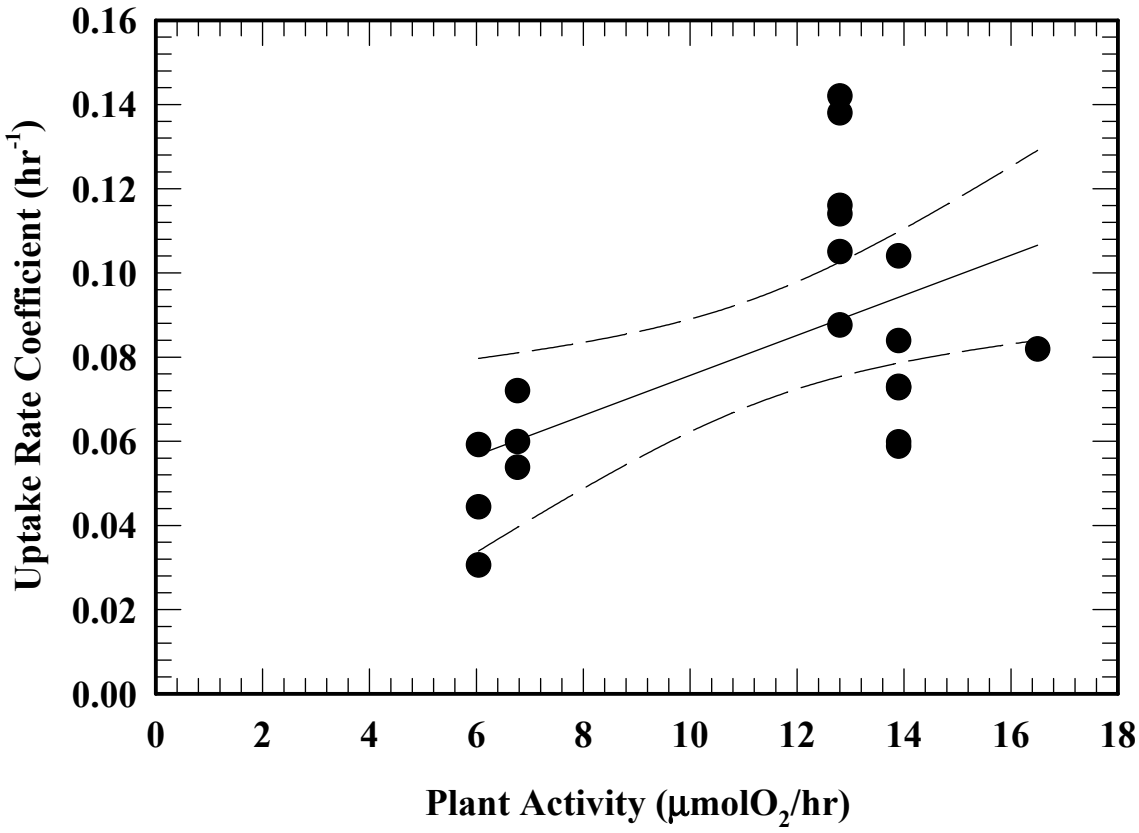


Figure 6.9. Plant activity, α, and first-order contaminant uptake rate coefficients shown for pH 6 with 2,4,5-TCP at 0.46 – 2.59 mg/L. A linear regression line and 95% confidence intervals are shown.

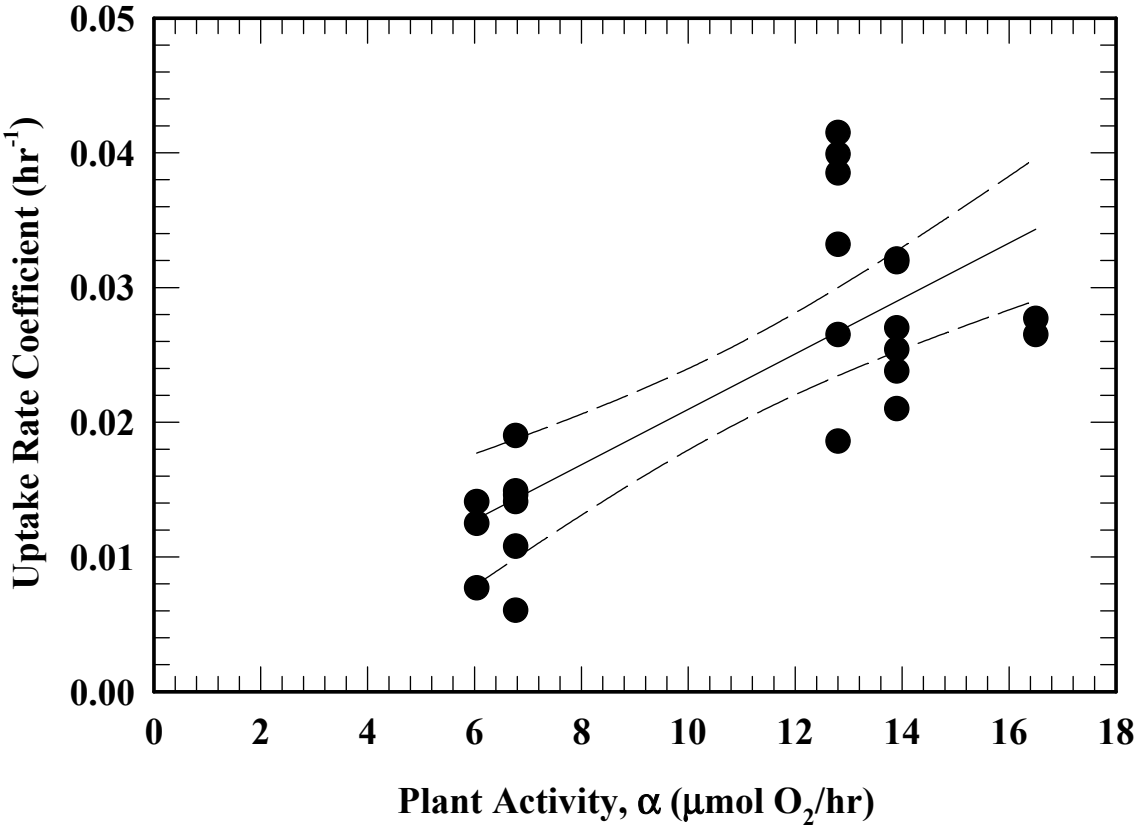


Figure 6.10. Plant activity, α, and first-order contaminant uptake rate coefficients shown for pH 8 with 2,4,5-TCP at 0.5 - 7.8 mg/L. A linear regression line and 95% confidence intervals are shown.

(0.0004 ± 0.0045) was not statistically different from zero. However, the y-intercept value for the pH 6 system was not equal to zero (0.0278 ± 0.0213), a result which was assumed to be a product of variation in data and not an indication that contaminant uptake occurs when plants are inactive. The slope for pH 6 systems was 4.7669 (±1.798) and the slope for pH 8 system was 2.056 (±0.3912). The t-statistic generated p-values < 0.0001 for the slope parameters, indicating that the slopes were known to be non-zero and therefore, the 2,4,5-TCP uptake rate was shown to depend on plant activity in systems with media pH of 6 and 8. Finally, the positive correlation further indicated that a relationship existed between plant metabolic activity and contaminant uptake and assimilation. However the linear relationships shown were not used in further data analyses except as a conceptual basis for the dependence of uptake rate on plant metabolic activity.

pH Dependant Contaminant Uptake.

Contaminant uptake was found to be accelerated as aqueous-phase pH values were decreased and other parameters were constant. This result was repeatedly demonstrated in uninhibited systems with the same initial 2,4,5-TCP concentration and media at various pH values. Aqueous-phase 2,4,5-TCP concentrations decreased, for example, considerably faster in pH 6 systems than in pH 8 systems. These results were demonstrated in Figure 6.5 where contaminant uptake rate was significantly faster in the pH 6 system than in the pH 8 system (i.e., k values were 2.47E-2 ± 1.86E-3 hr-1 and 6.07E-2 ± 3.89E-4 for pH 8 and pH 6 systems, respectively) even though initial concentration and other system parameters were identical in the two systems. To

investigate pH effects in detail, an experimental matrix was used that contained a range of initial 2,4,5-TCP concentrations, a range of α values and media pH values of 6, 7, 8 and 9. The pH values were selected based on a pK_a value of 7.0 for 2,4,5-TCP. This pH range was reasonable for typical environmental conditions and provided the opportunity to consider the effects of speciation on uptake rate. The pH range of 6 to 9 gave conditions with fraction of 2,4,5-TCP in its protonated form (i.e., $f = (K_a/[H^+] + 1)^{-1}$) of 0.909, 0.50, 0.091 and 0.0010.

First-order uptake rate coefficients, k, were found for 2,4,5-TCP removal from the aqueous phase where systems were known to be uninhibited as per inspection of α values. Based on the observation that uptake of 2,4,5-TCP was related to plant activity, rate coefficients (k) were then normalized to the corresponding plant activity (α) to include plant activity in a normalized uptake rate, k/α. As reported previously, α varied 10-fold over the course of several years due to inherent variations in plant systems employed. In experimental observations gathered for examination of effects of contaminant speciation and plant activity on contaminant uptake rate, α varied 3-fold (i.e., 6.0 – 16.5 μmol/hr). Therefore, this normalization of uptake rate to α was used to address variation in plant metabolic activity and coupled uptake to plant metabolic activity. To address the error associated with normalization of k to α values, example data for k and α data are presented in Table 6.1 with the standard error associated with the non-linear regression for determination of k values and the standard deviation for associated α values. Relative standard deviation (RSD) values for k and α values were determined using standard error, standard deviation, and mean values presented in

Table 6.1. Example data for examination of error associated with normalization of k to α.

C_0 (mg/L)	pH	k x 10^3		α		$RSD_{k/\alpha}$ (%)
		hr^{-1}	std. error	μmol/hr	std. dev	
6.38	8	8.9	1.08	11.7	0.85	14.4
6.36	8	9.8	0.61	11.7	0.85	9.9
6.37	8	9.6	0.44	11.7	0.85	8.9
0.81	8	12.6	0.25	6.0	0.80	16.5
3.33	8	10.9	1.75	6.0	0.80	20.7
7.76	8	6.4	0.97	6.0	0.80	20.2
0.59	6	45.9	2.45	6.0	0.80	14.2
0.71	6	34.3	10.8	6.3	1.38	38.4
1.16	6	20.7	0.48	6.3	1.38	22.0
3.84	8	25.7	2.28	16.5	2.69	18.6
2.12	8	24.7	1.86	16.5	2.69	18.0
2.07	6	60.7	0.39	16.5	2.69	16.3

Table 6.1 (i.e., RSD_k and RSD_α were calculated for each example data point in Table 6.1). Relative standard deviations ($RSD_{k/\alpha}$) for the k/α values were calculated by taking the square root of the sum of the squares of the RSD values for k and α (i.e., $RSD_{k/\alpha} = (RSD_k^2 + RSD_\alpha^2)^{0.5}$. RSD values for k/a for example data shown in Table 6.1 ranged from 8.9 – 38.4%, indicating that variability for data presented was reasonable for combining independently parameters gathered with independent measurements and statistical assessments in biological systems.

To further explore the relationship existing between k and α, the normalized uptake rate (k/α) was compared to initial 2,4,5-TCP concentrations at which plants were exposed in Figure 6.11. Data in Figure 6.11 are delineated by pH of plant exposure media where clear evidence of a decrease in k/α with increasing pH was observed over an order of magnitude change in initial 2,4,5-TCP concentration (0.5–7.8 mg/L) in spite of the large amount of variability existing in data presented (i.e., high RSD values for k/α normalization). Normalized uptake rate coefficients were significantly different for each pH value, and pH stratification shown in Figure 6.11 demonstrated that normalized uptake rate coefficient were relatively constant within a given pH value. Normalized uptake rate coefficients for pH 7 systems exhibited a small decreasing trend as concentration increased which was attributed to subtle toxic effects that were not detected by plant activity measurement or to routine variability that exists in plant systems. Therefore, normalized uptake rate coefficients were projected as constants by averaging k/α values for each pH tested in order to examine the dependence of uptake rate on media pH and use data in further modeling application.

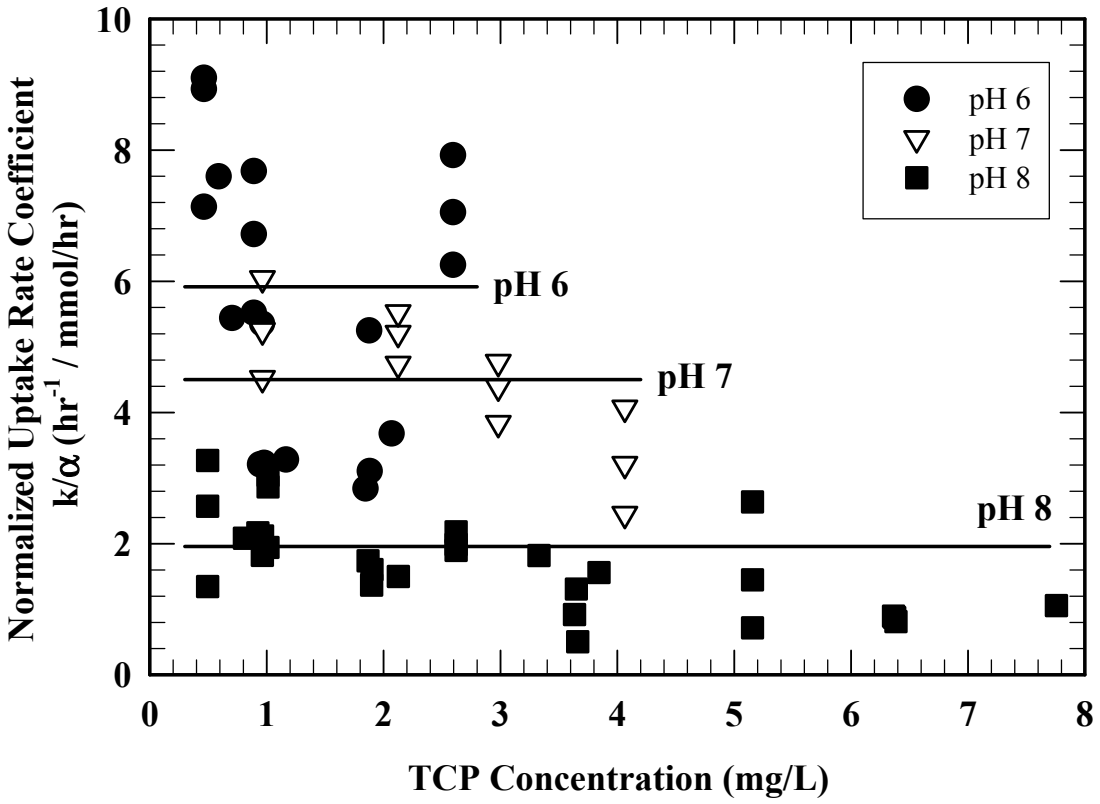


Figure 6.11. Uptake rate coefficient normalized to plant activity over 2,4,5-TCP concentration range of 0.5 to 7.8 mg/L. Average normalized uptake rate coefficients were 5.91 (±2.10) $mmol^{-1}$, 4.50 (±1.00) $mmol^{-1}$, and 1.96 (±1.32) $mmol^{-1}$, for pH values of 6, 7, and 8, respectively.

Average values for normalized uptake rate coefficients, k/α, for pH values of 6, 7 and 8 were 5.91 (±2.10) $mmol^{-1}$, 4.50 (±1.00) $mmol^{-1}$, and 1.96 (±1.32) $mmol^{-1}$, respectively. These results demonstrate a clear effect of pH on k/α values, which decreased as pH values increased. All data shown in Figure 6.11 represent systems that were uninhibited by 2,4,5-TCP as indicated with experimentally-measured α values throughout the experiment. In addition, data presented for pH 6 span a narrow range of initial concentrations as compared to data presented for pH 8 (e.g., 0.46 – 2.59 mg/L for pH 6; 0.49 – 7.75 mg/L for pH 8). This is because toxicity was observed at lower initial concentrations for lower pH values. For example, toxicity was observed at $C_0 >$ 2.59mg/L for pH 6 systems and these data were not included in Figure 6. The observation of toxic effects at lower initial concentrations in lower pH systems was an effect of the increased contaminant uptake rate. Increased mass loading into plant tissue was observed at low pH values therefore the contaminant body burden on plants at lower pH values was also increased, resulting in toxic effects observed at lower initial contaminant concentrations.

Increased contaminant uptake rate was observed at low pH values (i.e., k/α was inversely related to pH), a observation which was attributed to increased availability of the contaminant in the protonated form. The observation of increased contaminant uptake rate at low pH values was consistent with the concept that the protonated form of contaminant was the species available for partitioning into plants. Variations in pH 6 systems were greater than those of higher pH values, an effect which was attributed to the fact that 90.9% of the contaminant was in the protonated form and was consequently

highly available for partitioning into the plant. It is reasonable to conclude that pH 6 systems were more sensitive to changes in plant membranes, enzyme activity and other factors that play a role in controlling rate of contaminant uptake. The increased sensitivity observed in pH 6 systems was because contaminant was more readily available for partitioning than in higher pH systems and availability was not a limiting factor. Therefore, normalized uptake rate coefficients were projected as constant values over an order of magnitude range of initial concentrations and appropriately represented as average values of normalized uptake rate coefficients for each pH value.

Average values of normalized uptake rate coefficients (k/α) in Figure 6.11 were examined as a function of fraction of 2,4,5-TCP in the protonated form (i.e., fraction protonated, $f = [(K_a /[H^+]+1)^{-1}]$) because the protonated form was considered to be the species available for partitioning into plants. As fraction of contaminant in the protonated form decreased, the k/α values decreased (Figure 6.12), further demonstrating the relationship observed for increased uptake rate at lower pH values. A relationship existed between k/α and fraction of contaminant in the protonated form over an order of magnitude of initial 2,4,5-TCP concentrations (i.e., 0.46 – 7.75 mg/L) where k/α values increased with increasing fraction of contaminant in protonated form. These data indicated that mass transfer into the plant was dependant on amount of aqueous-phase contaminant in the protonated form. In this regard, the pK_a value for 2,4,5-TCP was 7.0, indicating that protonated 2,4,5-TCP was the dominant form of 2,4,5-TCP for pH 6 systems where increased uptake rates were observed. Similarly, unprotonated 2,4,5-TCP was the dominant form in pH 8 systems where reduced uptake rates were observed. In

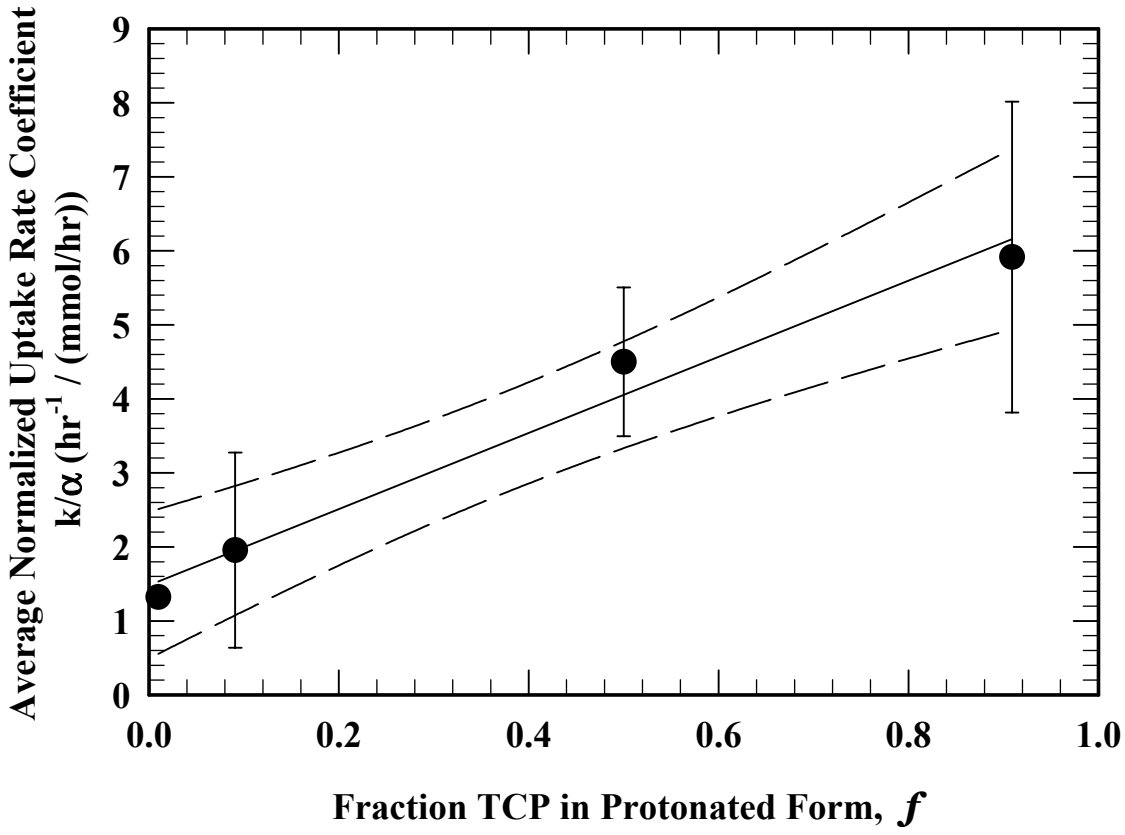


Figure 6.12. Correlation between normalized plant uptake rate (k/α) and fraction of 2,4,5-TCP in the protonated form (f). The linear regression ($\kappa = k/\alpha = 1.48\ (\pm 0.28) + 5.15\ (\pm 0.54)\ f$; $R^2 = 0.98$) was used to define the parameter κ and is shown with 95% confidence intervals. The t-statistic produced two sided p-values < 0.05, indicating that both the slope and intercept parameters were valid.

further examination of this linear relationship, a linear regression was used to describe data in Figure 6.12 which provided an R^2 value of 0.98, as shown with 95% confidence intervals. The t-statistic for the slope parameter generated a p-value < 0.0001, indicating that the slope parameter was known to be non-zero. The parameter kappa (κ) has been used to define the relationship between fraction of contaminant in the protonated form (*f*) and k/α, as was characterized by the linear regression in Figure 6.12 (i.e., $\kappa = k/\alpha = 1.48 (\pm 0.28) + 5.15 (\pm 0.54)f$). The parameter κ, with units of hr^{-1}/(mmol/hr), defined contaminant uptake rate as a function of α and fraction of contaminant in the protonated form and can be used to examine contaminant uptake for a range of α, pH and initial concentrations in systems where toxicity was not observed. Thus, normalized uptake rate coefficient, k/α, was shown to depend on fraction of contaminant in protonated form and was independent of initial concentration.

Discussion

Relationships have been developed that demonstrated dependence of contaminant uptake rate on plant metabolic activity and contaminant speciation. The relationships developed demonstrate the need to independently assess plant activity in studies of environmental fate of contaminants and phytoremediation of contaminants because they are critical variable affecting contaminant uptake rate. In addition, data for inhibited systems demonstrated a clear reduction of contaminant uptake with time and a decrease in plant activity with increased contaminant accumulation rate. Furthermore, contaminant uptake for most xenobiotics (including 2,4,5-TCP) is consistent with a passive process that is

driven by abiotic partitioning into the organic material comprising the plant and is supported by the correlation of k/α with f in our studies.

The partitioning behavior of 2,4,5-TCP in plant systems can be further explored by examining relationships for soil systems with high organic content. pH-based partitioning behavior for 2,4,5-TCP in plant systems is consistent with the concept that the extent of sorption in soil systems varies as a function of various solution properties, such as pH as was outlined by Schwarzenbach and co-workers (1993). Fragment constants both forms of 2,4,5-TCP were used to estimate organic matter-water partition coefficients for protonated and unprotonated forms of 2,4,5-TCP where the protonated form had greater affinity for organic matter than the unprotonated form thus demonstrating that protonated 2,4,5-TCP was the primary form of contaminant active in sorption in soil systems. Similarly, it can be assumed that partitioning into plant was driven by the protonated form of the contaminant. Therefore, the protonated form of 2,4,5-TCP was the major form of the contaminant moving into the plant and contaminant uptake was driven by the fraction of the contaminant in the protonated form. Results given in the development of the parameter κ, which showed linear dependence of contaminant uptake rate on fraction of contaminant in the protonated form, support conclusions regarding the passive nature of contaminant uptake by plants. Previous studies have also indicated contaminant uptake to be a partitioning process not regulated by the plant (Paterson et al., 1994; Trapp et al., 1990; Trapp and McFarlane, 1995).

The demonstrated link between uptake rate and plant metabolic activity indicated that contaminant uptake is a process actively influenced by plant activity. Results however also showed that contaminant uptake was driven by the protonated form, indicating that contaminant uptake was an abiotic, passive process and not actively driven by the plant. This inconsistency can be reconciled by considering abiotic partitioning and enzymatic processing in series. In addressing this inconsistency, abiotic partitioning of contaminant into plants was defined by the term κ and equilibrium sorption to surfaces of plants was defined by a Freundlich isotherm (Chapter 4). After the protonated form of the contaminant has partitioned into the plant, the contaminant is then enzymatically processed and packaged to reduce toxicity. When the rate of partitioning exceeded the rate of enzymatic contaminant processing, a contaminant pool developed internal to the plant and the rate of contaminant partitioning into plants was decreased. The effects of increased uptake rates forming a projected internal pool of contaminant was observed in low pH systems where rapid uptake caused toxic effects. In higher pH systems, slower uptake allowed systems to remain uninhibited because plants could enzymatically process the contaminant at a rate which did not allow for internal accumulation of the contaminant to toxic levels. Systems with decreased uptake rates often accumulated more mass than those with faster uptake rates because the contaminant did not reach elevated concentrations internal to the plant and did not thereby cause toxic effects. Thus, the overall processing of contaminants by plants relies both on abiotic processes, where factors such as protonation must be considered, and on metabolically-driven factors where plant activity must be considered.

CHAPTER 7

A CONCEPTUAL MODEL FOR CONTAMINANT UPTAKE BY AQUATIC PLANTS INCORPORATING INHIBITION, PLANT ACTIVITY, AND CONTAMINANT SPECIATION

Organic contaminants are known to have inhibitory effects on plants and other aquatic organisms. Standard toxicity tests are used to identify contaminants of concern for human health, as well as assess risks to wildlife. Many legislative guidelines have included standard toxicity tests with plant species as a part of environmental monitoring and assessment (Wang and Freemark, 1995). Complex effluents that are released into aquatic environments, such as effluents from municipal and industrial facilities, including petroleum refineries, pulp and paper mills, wastewater treatment plants, and landfills, are commonly screened using whole effluent testing (WET) and other standard toxicity tests (Wang and Freemark, 1995; Maltby *et al.,* 2000; Nwosu *et al.,* 1991; Bailey *et al.,* 2000; Sarakinos, 2000). The floating vascular aquatic plant, *Lemna minor,* is a frequently-used specimen for standard toxicity testing and WET with test procedures outlined in Method 8211 (Greenberg *et al.,* 1998). Because of its wide use as a test species for toxicity assessment, numerous data are available for quantifying toxic effects of contaminants in *L. minor* systems.

There is a need for enhanced understanding of how metabolic activity of plants affects transformation of contaminants. In a recent review of phytoremediation of organic pollutants, Trapp and Karlson (2001) identified the need for assessing phytotoxicity and addressing contaminant dosage in terms of toxic effects. Conceptual models that describe and predict uptake and metabolic processing of organic contaminants by plants are required to appropriately demonstrate the critical roles that aquatic plants play in attenuation of contaminants in the environment and to enhance the engineered use of plants to mitigate contamination.

Trapp and McFarlane (1995) modeled contaminant interaction in terrestrial plant systems including parameterized processes such as diffusive exchange between soil and roots in water and air pores, translocation in plants with transpiration streams, partitioning into stems, diffusive exchange between air and leaf via stomata and cuticle, metabolism, and dilution by growth. In the fugacity-based model outlined by Trapp and McFarlane (1995), partitioning between plant and aqueous phases was driven by hydrophobicity of contaminants and ionizable contaminants were excluded from their approach. A model proposed by Hattink and co-workers (2000) described interactions between *L. minor* and technetium-99 involving first-order partitioning into plants and first-order enzymatic reduction of the radionuclide internal to plants. However, this previous work did not consider sorption to plants, plant activity effects, water-column properties or other relevant variables.

Gobas and co-workers (1991) examined partitioning between aqueous and plant phases for tri- to hexa-chlorinated benzenes and biphenyls (log K_{ow} values: 4.02 – 8.26) with low initial concentrations ranging from 0.072 to 5.92 μg/L. A flow-through system was used with the aquatic macrophyte *Myriophyllum spicatum* where contaminant uptake over 25 d and contaminant elimination over 133 d were monitored. Contaminant transformation by plants was not observed or examined. Wolf and co-workers (1991) investigated a diffusion based model of uptake, sorption and bioconcentration by submerged plants through comparison between active and inactive plants. Contaminant accumulation results were similar for active and inactivated shoots for the first 3 hr of exposure, after which inactivated shoots accumulated significantly more contaminant than fresh shoots. These results indicated that other processes such as contaminant metabolism were occurring in active plant systems.

Incorporation of plant activity and toxic effects of contaminants into a model that outlines plant-contaminant interactions provides a robust and meaningful description and prediction of fate of contaminants in plant systems. The model presented here was developed using *L. minor* as the model aquatic plant and 2,4,5-trichlorophenol (2,4,5-TCP) as the model contaminant. This experimentally-coupled modeling effort was a novel examination of plant interactions with an organic contaminant which is ionizable at biologically relevant pH values (i.e., $pK_a = 7.0$). This contribution provides an innovative examination of effects of plant activity and inhibition on rates of contaminant uptake and enzymatic processing. A conceptual model is presented for aquatic plants that integrates uptake of contaminants by plants, contaminant sorption to plants, and

metabolic processing of contaminants by aquatic plants with metabolic activity of plants. Inhibition of plant metabolic activity caused by internal contaminant accumulation of untransformed contaminants was incorporated.

Effects of Inhibition of on Uptake of 2,4,5-TCP by *L. minor*

A reliable method for determining inhibition was necessary to gauge effects of contaminants on plant metabolic activity and to link inhibition to contaminant uptake rate. Plant activity measurements were taken before exposure to contaminant to gauge background plant metabolic activity and plant activity measurements taken after exposure to contaminants were used to assess inhibition of plant systems by contaminants or external factors.

Plant Activity and Inhibition (α, β).

Oxygen production rate has been used to measure photosynthetic activity for use as an indicator of plant activity (α). Control-plant oxygen production rates were used as an indicator of background plant activity since variations in stock plant activity were commonly seen with fluctuations in nutrient loading, light availability, temperature, and other weather and seasonally dependant factors. Relative plant activity (β) was used to assess inhibitory effects of contaminants at sampling time points during experimentation (e.g., t = 2, 24, and 48 hr). Relative plant activity, β, was defined as the oxygen production rate of a 2,4,5-TCP-exposed system at any given time point through an experiment ($\alpha_{t=i}$) normalized to oxygen production rate of a non-contaminated control ($\alpha_{t=0}$) therefore $\beta_{t=i} = \alpha_{t=i} / \alpha_{t=0}$. When plants were not inhibited, $\alpha_{t=i} = \alpha_{t=0}$ and therefore,

$\beta = 1$. When plants were inhibited, oxygen rate decreased and $\alpha_{t=i}$ was less than $\alpha_{t=0}$ and $\beta < 1$. Plants were considered to be fully inhibited when no oxygen was produced by plants ($\alpha_{t=i} = 0$) and $\beta = 0$.

An illustrative example of relative plant activity (β) measurements for plants inhibited by 2,4,5-TCP is presented in Figure 7.1. Data presented represent triplicate reactors of 2,4,5-TCP treated plants and control plants (i.e., control plants underwent the same treatment as 2,4,5-TCP-treated plants without contaminant exposure) which were sacrificed after 0, 24 and 48 hr of exposure. In the control systems presented in Figure 7.1, oxygen production rate was continuous over 48 hr for plant exposed in media fixed at pH values of 6 and 8, demonstrating that plant metabolic activity was constant with time. Plants treated with high concentrations of 2,4,5-TCP were inhibited by the contaminant as exhibited by declining and zero values of relative plant activity (β). In Figure 7.1, $\beta = 0.65$ after 2 hr, and $\beta = 0.59$ after 24 hr indicating that plants were partially inhibited by 2,4,5-TCP after 2 hr of exposure and plant were increasingly inhibited by 2,4,5-TCP 24 hr of exposure. After 48 hr of exposure, $\beta = 0.02$, indicating that plants were fully inhibited by 2,4,5-TCP and no photosynthetic processes were occurring.

Relative Plant Activity and Uptake of 2,4,5-TCP.

Relative plant activity measurements were taken in conjunction with aqueous 2,4,5-TCP measurements to explore effects of inhibition on 2,4,5-TCP uptake. 2,4,5-TCP was eliminated from the aqueous phase in a first-order rate when oxygen production rate was

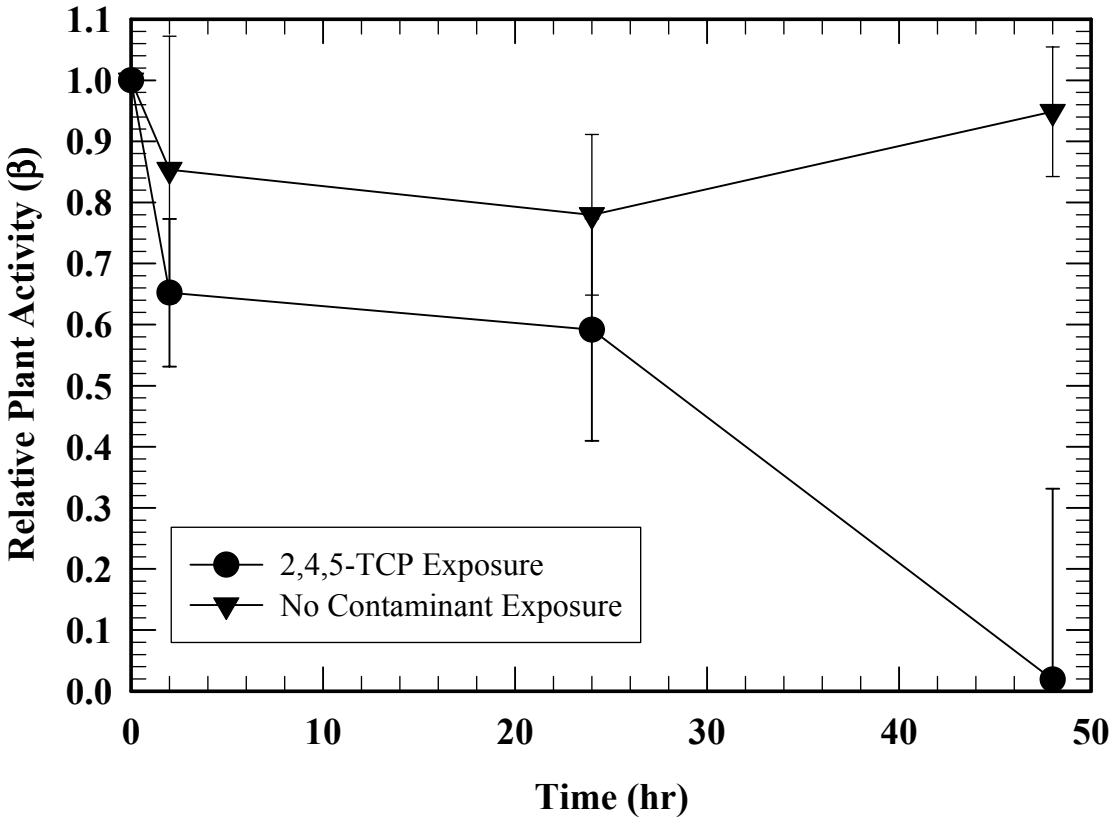


Figure 7.1. Example relative plant activity (β) data for *L. minor* after 2,4,5-TCP exposure (11.7 mg/L) and in a no-2,4,5-TCP control with media pH values of 8.

similar to that of live control (i.e., $\beta = 1$ or no inhibition was apparent). Where plants showed a decreased contaminant uptake rate, a corresponding decline in β occurred. When β was zero, signaling plant inactivation or death, 2,4,5-TCP removal ceased. Example data and first-order rate projections for systems where $\beta = 1$ throughout an experiment and where β decreased and dropped to near zero values over a 48 hr experiment are given in Figure 7.2. Rapid 2,4,5-TCP uptake occurred in systems where α values were unaffected (i.e., $\beta = 1$). Decreased or zero oxygen production ($\beta < 1$) dictated declining 2,4,5-TCP uptake rates, development of plateau 2,4,5-TCP concentrations, and reduced contaminant assimilation.

Data representing systems where inhibition was observed ($\beta < 1$) are presented in Figures 7.3 – 7.5. Figure 7.3 represents an aqueous concentration profile of 2,4,5-TCP with time and corresponding oxygen production rate measurements of plants exposed to 4 mg/L 2,4,5-TCP at pH values of 6 and 8. In the pH 6 system, partial inhibition was observed at 24 hr ($\beta = 0.24$) and nearly complete inhibition was observed by 48 hr ($\beta = 0.09$). 2,4,5-TCP profiles reflected this inhibition with decreasing rates by 24 hr and plateau concentrations reached at later time points of the experiment. Relative plant activity for the pH 8 systems presented in Figure 7.3 indicated that plants were partially inhibited after 24 hr of exposure to 2,4,5-TCP ($\beta = 0.40$), but regained activity by 48 hr and were minimally inhibited at the end of the experiment ($\beta = 0.78$). Inhibition was a result of contaminant initially pooling internal to plans at inhibitory levels pools and subsequent recovery of full activity is a result of enzymatic processing of contaminants by plants as is discussed in subsequent sections.

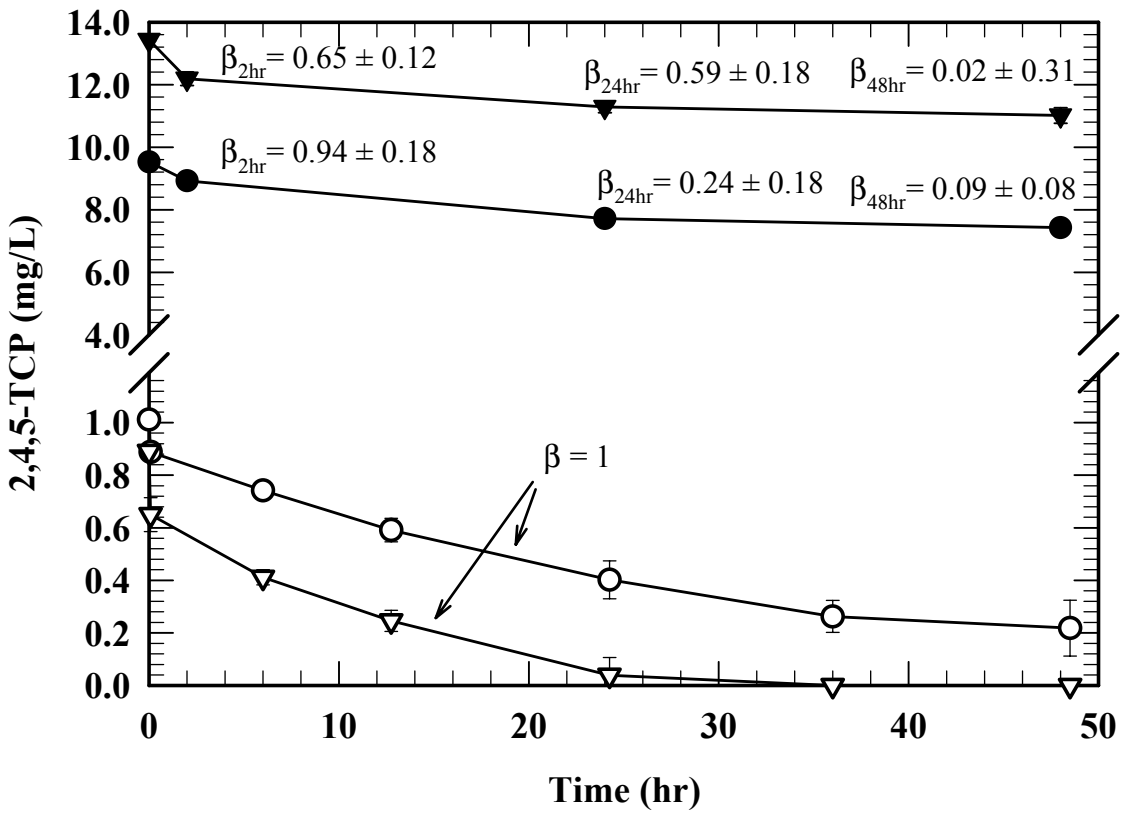


Figure 7.2. Aqueous phase 2,4,5-TCP in experimental plant systems in which plant inhibition (i.e., $\beta = 0$) resulted in termination of 2,4,5-TCP uptake. Values for relative plant activity (β) provided to give evidence of system inhibition. Data shown represent systems with various pH values, initial 2,4,5-TCP concentrations, and initial plant activities.

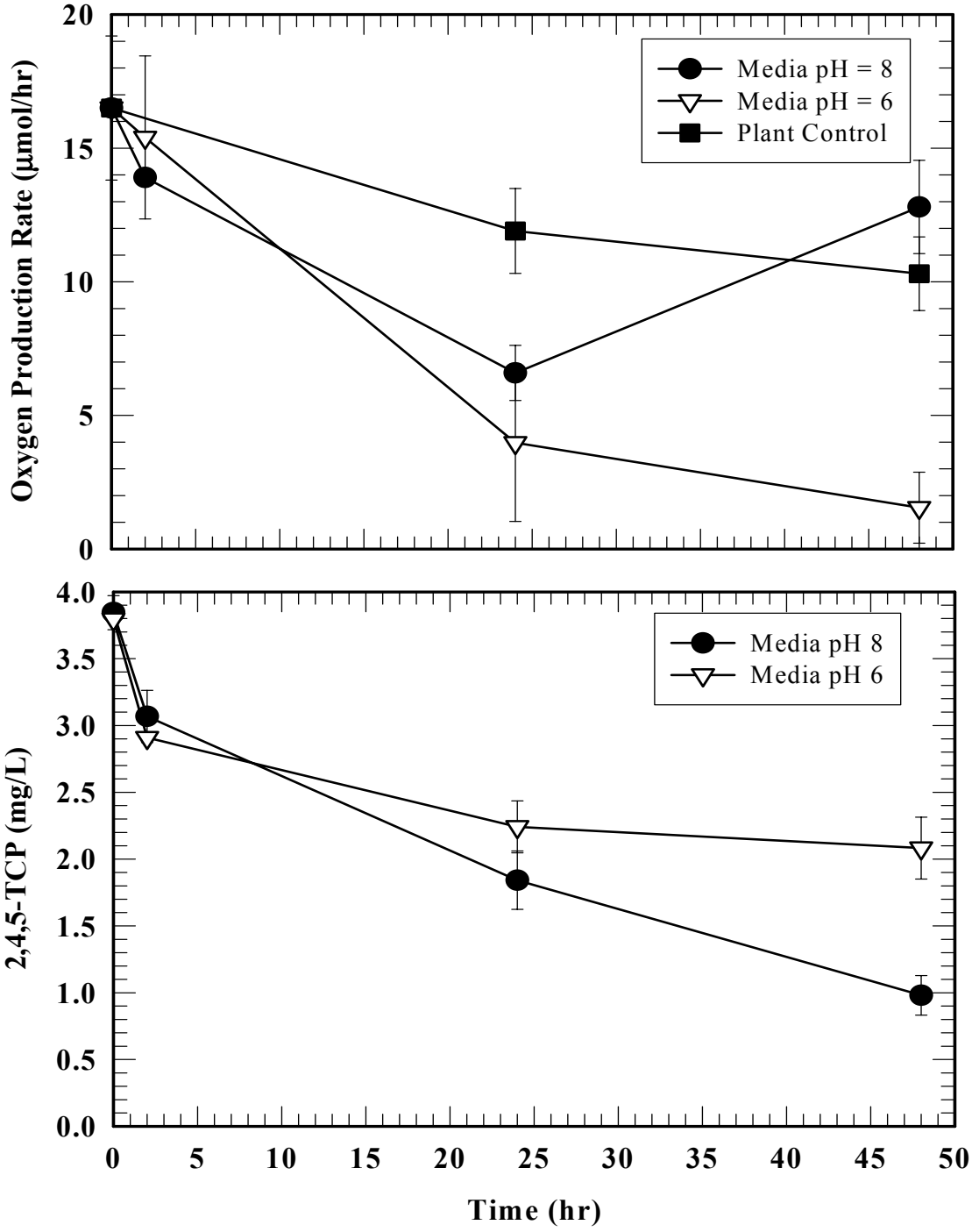


Figure 7.3 Aqueous 2,4,5-TCP coupled with oxygen production rate measurements and β values. 2,4,5-TCP uptake rate declined with $\beta < 1$.

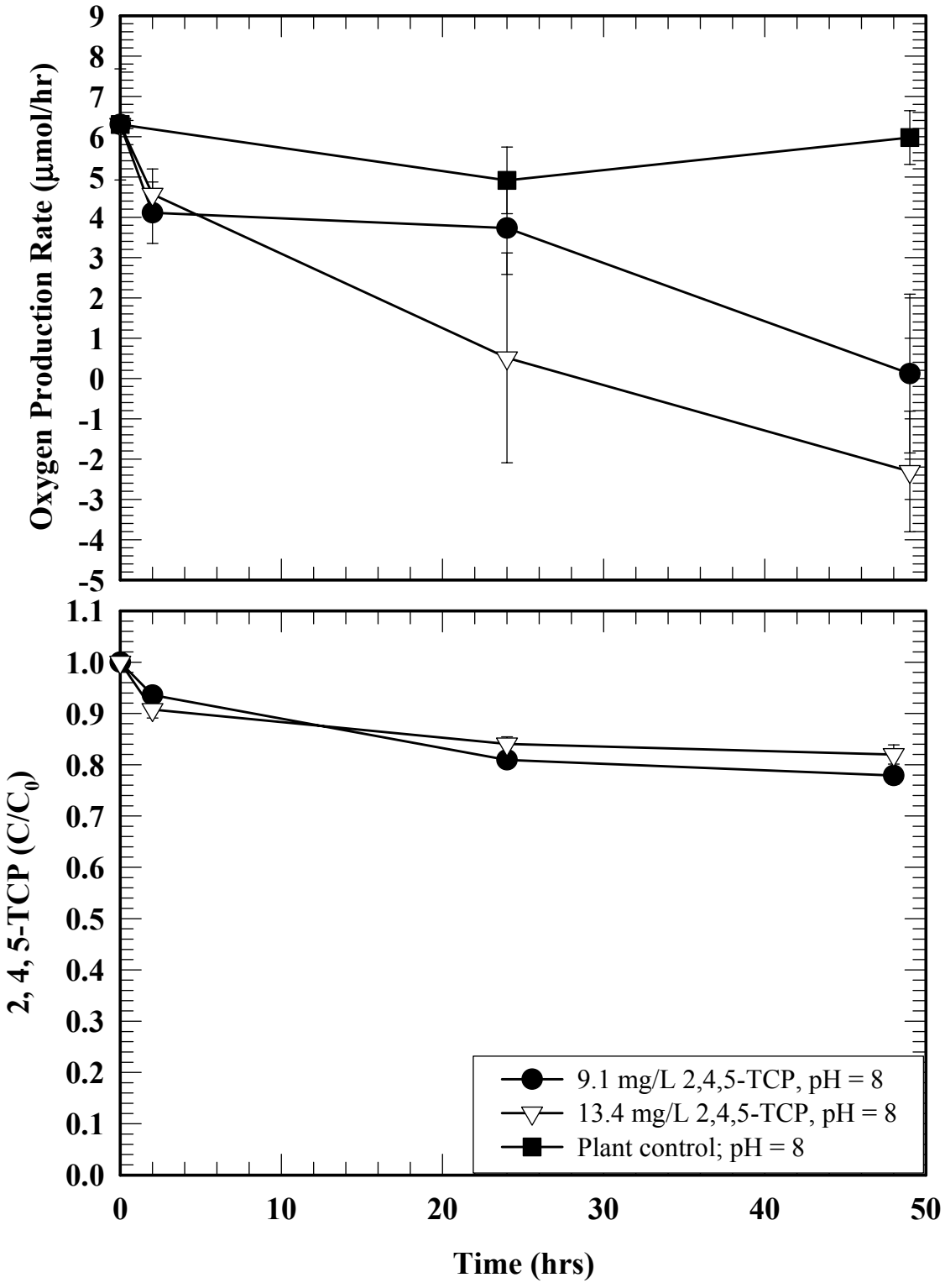


Figure 7.4. Inhibition of plants exposed to 2,4,5-TCP at 9.1 and 13.7 mg/L. 2,4,5-TCP uptake rate declined with $\beta < 1$.

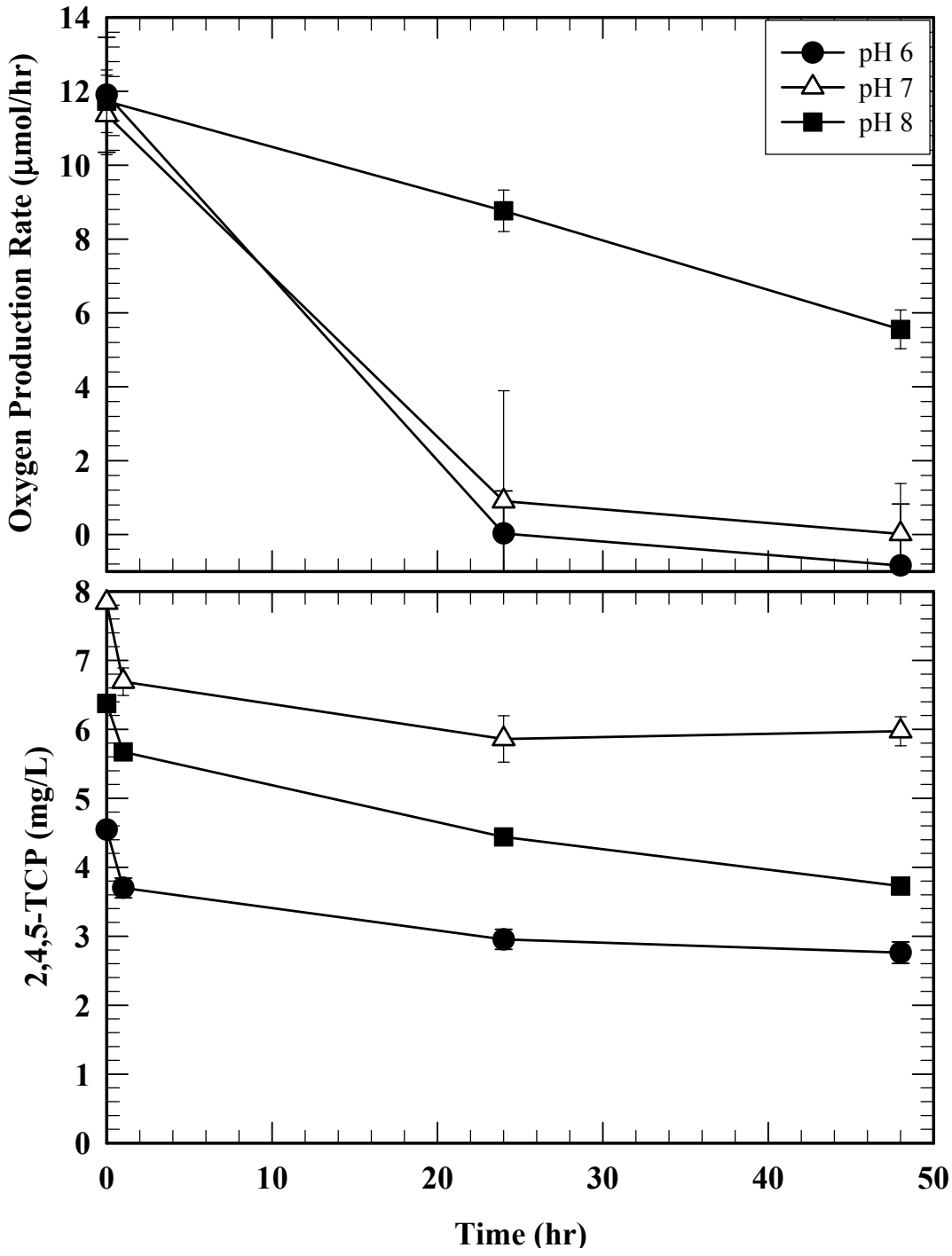


Figure 7.5. Plant exposed to three concentrations of 2,4,5-TCP at pH values of 6, 7 and 8. 2,4,5-TCP uptake rate declined with $\beta < 1$.

Figure 7.4 represents plants exposed to 9.1 and 13.4 mg/L of 2,4,5-TCP at a pH value of 8. Data show that plants were increasingly inhibited with time. In corresponding aqueous phase measurements, 2,4,5-TCP uptake declined and a plateau concentration was reached in both systems. Data presented in Figure 7.5 represents plants exposed to 4.8, 7.8 and 6.4 mg/L of 2,4,5-TCP at media pH values of 6, 7, and 8 respectively. Oxygen production rate data indicated that pH 6 and pH 7 systems were fully inhibited by the end of the experiment where β values were -0.07 and 0.00, respectively. 2,4,5-TCP measurements for pH 6 and pH 7 systems indicated a decrease in contaminant uptake rate and plateau values reached in both systems. The pH 8 systems shown was partially inhibited during the experiment, with $\beta = 0.47$ after 48 hr of exposure. Contaminant uptake proceeded throughout the course of the experiment in pH 8 systems, however the contaminant uptake rate was not rapid because of partial inhibition observed and limitations due the small fraction of contaminant in protonated phase. It is important to note that the initial concentration of the system exposed at media pH of 8 was higher than that of the system exposed at a pH value of 6, where full inhibition was observed in the pH 6 system and only partial inhibition was observed in the pH 8 system.

Batch experiments presented in Figures 7.3 – 7.5 and those presented previously in Figures 6.7 – 6.9 were conducted at various concentrations of 2,4,5-TCP and oxygen production rate was measured at several time points (t = 2 – 48hr) after contaminant addition as was presented. These data (Figures 6.7 – 6.9 and 7.3 – 7.5) included measurements for a wide range of (i) control-plant activity responses, (ii) initial contaminant concentrations and (iii) pH values, all at a fixed plant mass of 2 g per 130mL

(i.e., plant mass = 15.4 g FW/L). A wide range of β values were measured, with observations of no inhibition (β ~ 1), partial inhibition (β ~ 0.3 – 0.7) and full inhibition (β ~ 0). Total 2,4,5-TCP removed from aqueous phase was the Plant Bioconcentration Factor (PBCF) with units of μg 2,4,5-TCP per g of plant. Results from work with ^{14}C-2,4,5-TCP (Chapter 5) demonstrated that contaminant removed from aqueous phase was sequestered in plants, therefore PBCF was calculated as the 2,4,5-TCP removed from aqueous phase. A comparison of PBCF with relative plant activity (β) revealed that complex relationships underlay toxic effects driven by contaminant mass internal to plants (Figure 7.6). Data analysis showed that toxic effects occurred in some plants that accumulated a mass of 2,4,5-TCP greater than approximately 100 μg/g, although the toxic threshold was not absolute. These data indicated that complex relationships underlay contaminant-driven toxicity and plant-contaminant interactions and were further examined.

Conceptual Model to Describe Effects of Inhibition on Contaminant Uptake

A conceptual model was developed to describe uptake of organic contaminant by plants. The model incorporated sorption to plant surfaces, pH and plant activity driven uptake and effects of inhibition. Model development was based on kinetic data acquired from *L. minor* systems which were uninhibited and inhibited by 2,4,5-TCP as demonstrated experimentally with α measurements.

The conceptual model for ionizable contaminant uptake and processing by aquatic plants is illustrated in Figure 7.7 with a box diagram outlining contaminant interactions in

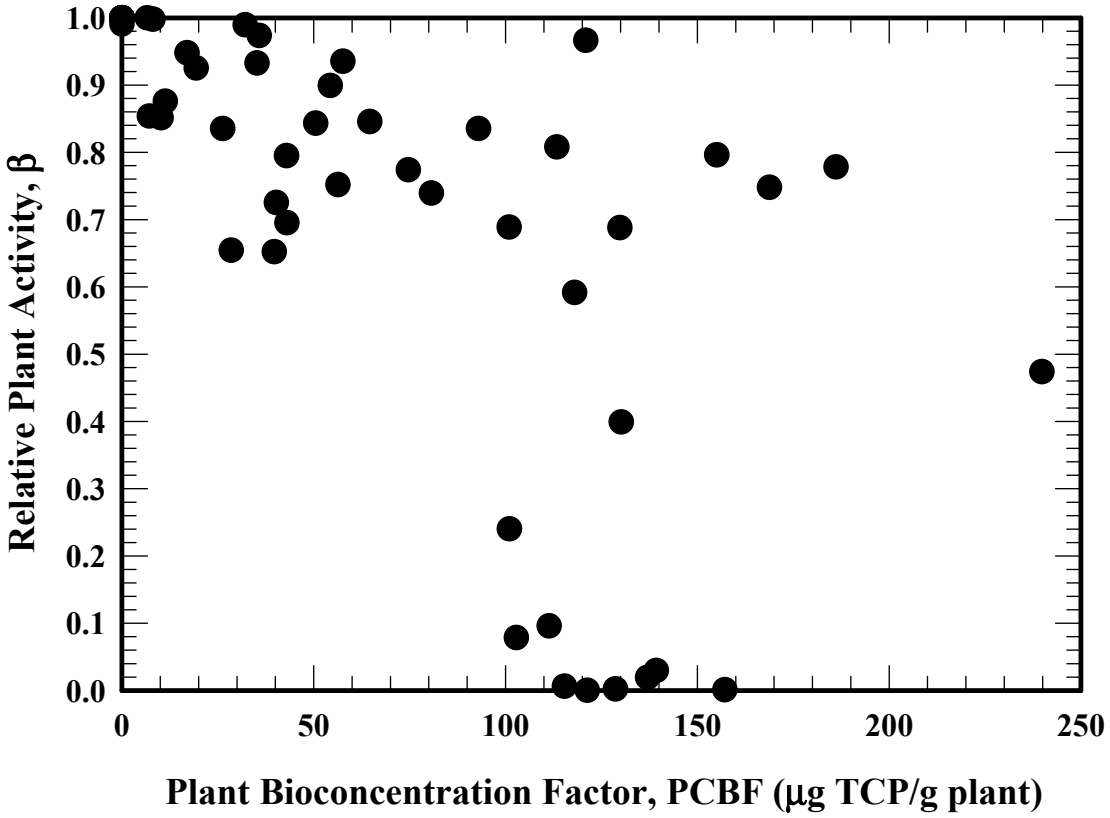


Figure 7.6. 2,4,5-TCP mass removed from aqueous phase compared with measured values of relative plant activity (β). Systems shown represent several time points after exposure and a variety of initial 2,4,5-TCP concentrations, pH values and plant activities. Relative plant activity (β) ranged from no inhibition (β = 1) to fully inhibited (β = 0).

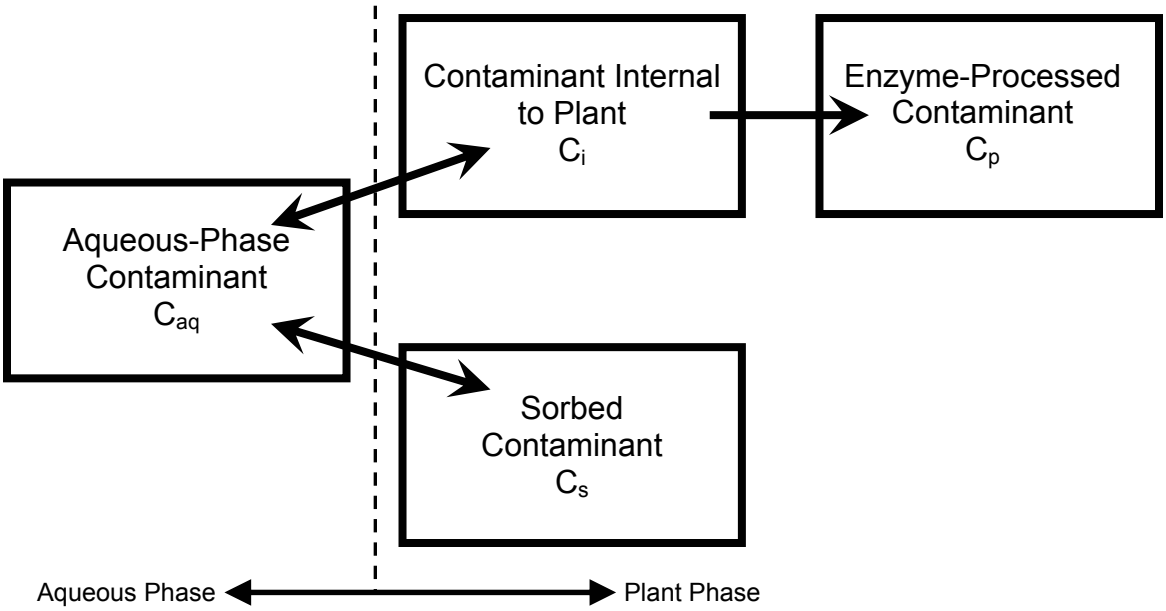


Figure 7.7. Contaminant interactions in *L. minor* with C representing a contaminant mass and subscripts used to identify aqueous phase (aq), internal, unaltered-contaminant (i), sorbed contaminant (s) and enzymatically-altered contaminant (p). Sorption and movement into plants are shown to be reversible processes, while plant-enzymatic processing represented an irreversible process.

L. minor. Aqueous and plant phases were established and contaminant forms of C_{aq}, C_i, C_s, and C_p were defined in mass units, where C_{aq} was aqueous-phase contaminant, C_i was contaminant internal to plants, C_s was contaminant sorbed to plants and C_p was enzymatically-processed contaminant. This conceptual model does not include atmospheric exchange because of the non-volatile nature of 2,4,5-TCP, as confirmed with experimental data for ^{14}C-2,4,5-TCP (Chapter 5). Growth-dilution was also not considered due to the relatively short duration of experiments modeled (~48hr) compared to the turnover times (5-7d) for *L. minor* as measured in our laboratories.

Contaminant Uptake, pH, and Plant Activity.

Experimental data from inhibited systems (previous section) and data for uninhibited systems presented previously (Chapter 6) provided the basis for the conceptual model presented herein. Results from uninhibited systems demonstrated that 2,4,5-TCP was rapidly removed from the aqueous phase in a first-order rate when plant activity (α) was similar to that of live control. A correlation was observed between α and contaminant uptake rate coefficient (k) in no-observed-effect systems, where increasing α values dictated increasing uptake rate coefficients for each given media pH value. Contaminant uptake rates coefficients (k) were normalized to plant activity values (α) to include plant activity in a normalized uptake rate, k/α.

Contaminant uptake was accelerated as aqueous phase pH values decreased over an order of magnitude change in initial contaminant concentration. The protonated form of contaminant was considered to be the species available for partitioning into plants;

therefore increased contaminant uptake rate observed at low pH values was attributed to increased availability of the contaminant. The effect of contaminant availability on contaminant uptake rate was further examined by comparing the average values of normalized uptake rates (k/α) for each pH value with the fraction of contaminant in the protonated form, f (i.e., $f = ((K_a/[H^+] +1)^{-1})$. This linear relationship between normalized uptake rate and extent of protonation was defined as κ was presented in Chapter 6 where a relationship of $\kappa = k/\alpha = 1.48 + 5.15f$ was developed, with f was the fraction protonated in aqueous phase and k was first order contaminant uptake rate for individual plant systems.

Contaminant Partitioning into Plants.

Contaminant uptake from aqueous phase has been described for aquatic plants as occurring through bulk water uptake, root growth and diffusion into root systems (Paterson *et al.,* 1994; Trapp *et al.,* 1990). Since *L. minor* sorb the majority of their water and nutrients through lower surfaces of fronds, and not roots, contaminant uptake was modeled as a foliar partitioning process (Landolt and Kandeler, 1987). In plant systems, uptake of certain nutrients and similar moieties can be an active process driven by trans-membrane transporter proteins that effectively increase nutrient concentration internal to plants to levels greater than naturally achieved by passive diffusion. However, for many nutrients and most contaminants, no transport mechanisms have been identified. It is clear that uptake of components such as micronutrients and contaminants is a passive process resulting from diffusion across plant membranes which is driven by concentration gradient between aqueous phase contaminant concentration and the

concentration of unaltered contaminant internal to plants. A mathematical description of diffusion of contaminants across the membrane into plants can be described using Fick's first law, a relationship which specifies that solute flux is proportional to the concentration gradient of the solute . However, the mathematical description of diffusion across a membrane in plant systems involves characterization of the concentration differential across the membrane. Characterization of the concentration gradient for ionizable contaminants in aquatic plant systems was complex because of where variables such as aqueous phase speciation, speciation internal to plants, rate of enzymatic processing, and plant activity play a significant role in altering concentrations in aqueous and plant phases. Therefore, contaminant uptake was described by non-equilibrium partitioning between the aqueous phase (C_{aq}) and the unaltered-contaminant internal to the plant (C_i) and the pseudo-first order rate coefficients developed previously (Chapter 6) were used to describe diffusive flux of contaminant into plants. The pseudo-first order rate coefficient used was representative of abiotic partitioning into plants and was a direct function of C_{aq}. As described in Figure 7.7, C_{aq} is inherently linked to C_i, and therefore, rate of contaminant uptake by plants was an indirect function of C_i. Description of contaminant uptake rate as a concentration dependant first-order process is consistent with previous descriptions of contaminant uptake by plants (e.g., Trapp and McFarlane, 1995; Hattnik et la., 2000). Because of the simplicity of the model aquatic plant used experimentally, this model does not specifically address internal chemical advection within plants, rather transport internal to the plant was considered to be taking place and taken into account by the parameters measured.

Enzymatic Contaminant Processing by Plants.

Contaminant processing and packaging by aquatic plants is an enzymatic reaction and has been shown to have a net accumulation of plant-produced metabolic products (e.g., Day, 2002; Day and Saunders, 2004; Hughes *et al.,* 1997; Roy and Hanninen, 1994; Ensley *et al.,* 1997). Therefore, contaminant processing by plants is presented as an irreversible process and biological processes that could return metabolic products and parent compounds to the aqueous phases were considered to be a part of the net rate of contaminant processing assessed herein. However, investigations have never demonstrated any release of contaminants into the aqueous phase from the plant phase, as was shown in Chapter 5 with ^{14}C data. In an effort to maintain simplicity, enzymatic processing of contaminants within plants was described by a first-order reaction, where C_p represented contaminant processed by enzymatic reactions internal to plants. A first-order rate was chosen over Michaelis-Menten kinetics to describe enzymatic processing to simply modeling with available experimental data. In this model, internal plant processing denotes sequestration, conjugation, oxidation, reduction, dechlorination, and any related processes that serve to detoxify contaminants or contaminant products. In the model outlined by Trapp and McFarlane (1995), production of C_p encompassed Phase I, II, and III processes facilitated by plants. Unaltered and untransformed contaminant, C_i, within plants was considered to be the toxic moiety and toxic effects associated with C_p, C_s, and C_{aq} were considered to be minimal and insignificant.

Prediction of Contaminant Uptake

The conceptual model outlined in Figure 7.7 is based on relationships presented in previous sections that defined plant-contaminant interactions in *L. minor* systems. Previously defined relationships that described contaminant uptake and sorption were incorporated into kinetic parameters, which were then merged with the conceptual model (Figure 7.7) to provide a mathematical description of plant-contaminant interactions which includes plant activity and relative inhibition. This model can be used to describe and predict contaminant fate in natural environment systems and phytoremediation processes.

Prediction of Contaminant Uptake Given Relative Inhibition (β) and Plant Activity (α).

The conceptual model was merged with relationships developed using previously presented experimental data to form a mathematical relationship that predicted contaminant uptake by plant systems. All data shown were gathered in reactors containing a fixed liquid volume (130 mL) and plant mass (2 g), therefore the model was developed on a mass basis where C_{aq} was mass of contaminant in aqueous phase, C_i was mass of unprocessed contaminant internal to plants, C_s was mass of contaminant sorbed to plant surfaces, and C_p was mass of enzymatically-processed contaminant internal to plants.

Contaminant sorption to plants was an extremely rapid process where equilibrium between aqueous and sorbed phases was reached in less than five minutes, as compared

to 24 – 48 hr period of contaminant exposure as was presented previously in Chapter 6. Data gathered in inactivated control systems to delineate sorptive behavior of 2,4,5-TCP in plant systems were fitted to a Freundlich isotherm (Figure 6.4). The Freundlich isotherm for 2,4,5-TCP in plant systems was developed for concentration of protonated 2,4,5-TCP (mg/L) and mass of sorbed 2,4,5-TCP (mg/g). The Freundlich isotherm for 2,4,5-TCP with *L. minor* was $q_e=0.023S^{0.55}$ where q_e and S were expressed as mg/g and mg/L, respectively. The Freundlich isotherm was used to maintain an equilibrium ratio between C_{aq} and C_s in the model, a concept consistent with rapid sorption and desorption. Isotherm parameters were normalized to plant mass (2 g) and media volume (130 mL) to provide consistent units. The rate of partitioning between C_{aq} and C_i was described by a first-order equation with respect to aqueous-phase contaminant, C_{aq}, in Equation 1 with a series of plant-related kinetic and activity parameters.

$$\frac{dC_{aq}}{dt} = -\kappa\alpha\beta C_{aq} \qquad (1)$$

From Figure 6.14, the kinetic parameter κ, where $\kappa = k/\alpha = 1.48\ (\pm 0.28) + 5.15\ (\pm 0.54)\,f$, was used to describe partitioning between aqueous and plant phases and was previously developed in Chapter 6. pH was incorporated into contaminant uptake rate by use of κ, describing contaminant partitioning between aqueous and plant phases. Because the uptake rate coefficient was normalized to plant activity in developing κ, it was necessary to multiply κ by plant activity (α) in Equation 1 such that the rate of partitioning would reflect the plant activity for the specific plant system being modeled. Therefore the product κα was a pseudo first-order rate coefficient for 2,4,5-TCP uptake for an uninhibited plant system (i.e., β = 1).

Because contaminant uptake was an active-plant mediated process, uptake rate was modified to reflect the activity of plants through time. The relative plant activity term, β, was used to modify reaction rate and to reflect a reduction in plant activity that would manifest itself as a reduction in contaminant uptake. As described previously, β was calculated as the plant activity of 2,4,5-TCP-treated plants at a given time in the course of an experiment normalized to the plant activity of control plants. If $\beta = 1$, contaminant uptake proceeded at rates dictated by background plant activity and level of protonated contaminant. When β decreased due to 2,4,5-TCP inhibition of plant systems, contaminant uptake was attenuated or eliminated. When β equaled zero, Equation 1 indicated no change in aqueous contaminant concentration, thus only the amount sorbed to external surfaces of plants was removed from the aqueous phase and the concentration profile reached a plateau value indicated by the linear sorption isotherm.

Equation 1 was used to predict contaminant uptake using initial contaminant mass in aqueous phase, α, κ, Freundlich sorption isotherm, and measured relative plant activity data (β). Example solutions of Equation 1 compared with measured data for corresponding systems are given in Figures 7.8 and 7.9. Predictions are shown for data gathered independent of kinetic parameter and model development. These projections predicted patterns of contaminant uptake in uninhibited plant systems and plant systems partially to fully inhibited through the time course of an experiment. Several pH values and background plant activities are included within the projections. These results

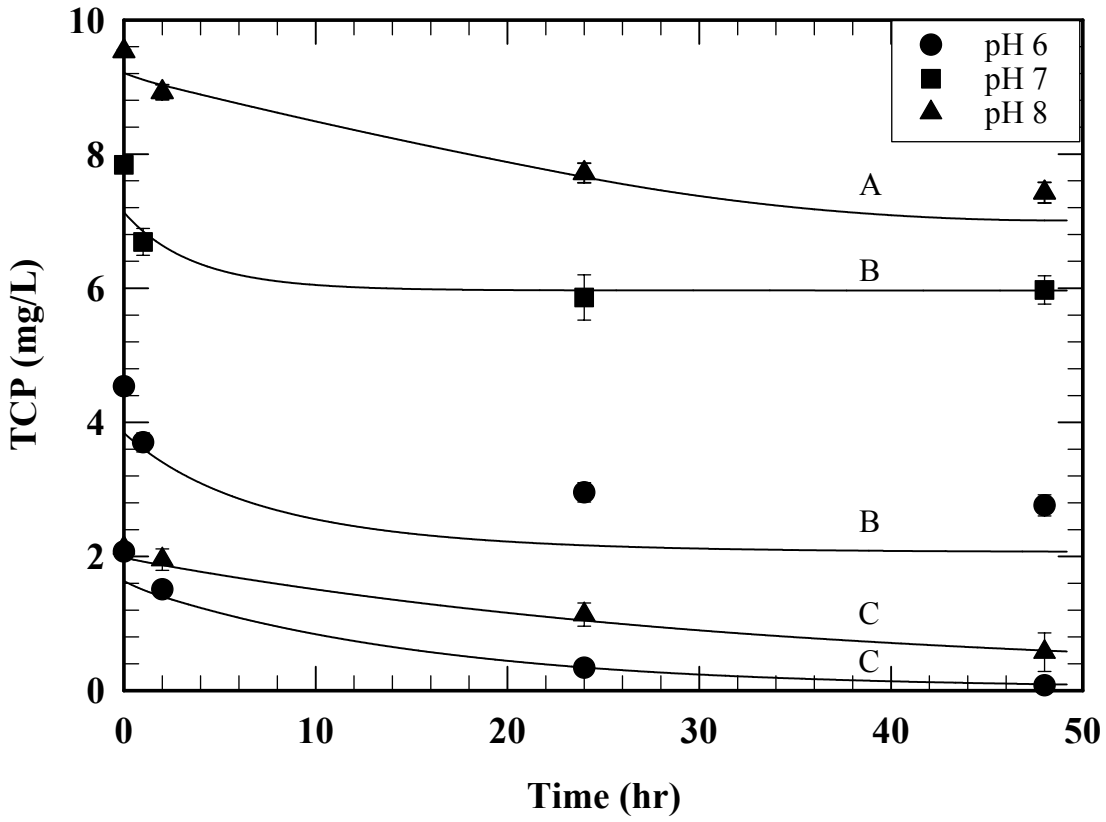


Figure 7.8. Prediction of contaminant uptake utilizing measured plant activity data. Example systems shown were: (A) partially inhibited, i.e., β decreased with time but did not reach zero; (B) fully inhibited, i.e., β=0 after initiation of experiment; or (C) uninhibited, i.e., β=1 over the time course of the experiment.

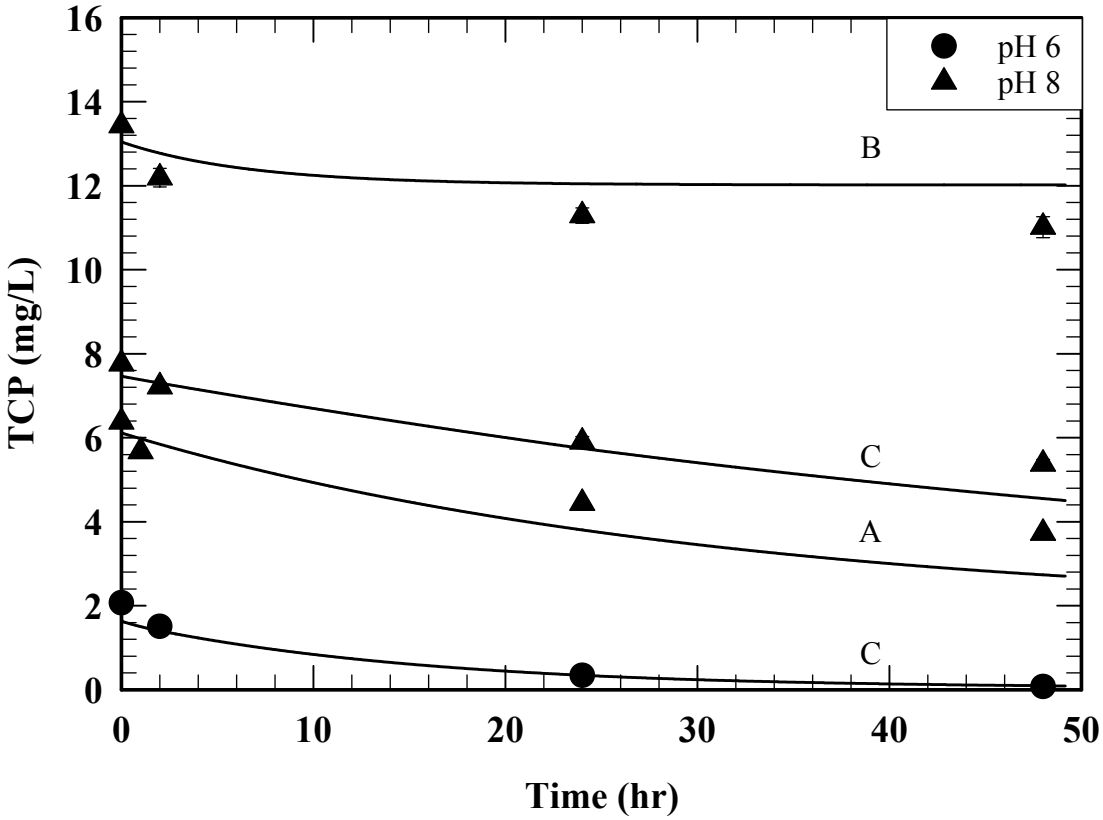


Figure 7.9. Prediction of contaminant uptake utilizing measured plant activity data. Example systems shown were: (A) partially inhibited, i.e., β decreased with time but did not reach zero; (B) fully inhibited, i.e., β=0 after initiation of experiment; or (C) uninhibited, i.e., β=1 over the time course of the experiment.

demonstrated that Equation 1 could be used to predict contaminant uptake for an ionizable contaminant in inhibited and non-inhibited systems.

Toxicity Relationship for Relative Plant Activity.

To predict contaminant uptake rate without explicit relative plant activity data through time, a relationship between relative plant activity, β, and the internal pool of contaminant, C_i, (i.e., the component considered to be toxic to plants) was developed. To develop a relationship between C_i and β, enzymatic processing of contaminants was modeled using a first order equation. The first-order relationship given in Equation 2 was used for the production of a non-toxic component, C_p, from the internal contaminant, C_i.

$$\frac{dC_p}{dt} = k_p \beta C_i \quad (2)$$

The rate coefficient, k_p (hr^{-1}) was used to describe rate of enzymatic processing of contaminant by plants. The relationship for enzymatic processing (Equation 2) was attenuated by the relative plant activity term, β, because it was a live-plant mediated process. C_i and C_p components were not measured explicitly, therefore rate of production of C_p was extrapolated from plant activity data and rate of contaminant removal from aqueous phase.

Values of k_p ranging from 0.05 to 0.5 hr^{-1} were used to solve Equations 1 and 2 in parallel for several data sets containing a range of initial conditions (α = 7.8 – 20.3 μmol/hr; initial 2,4,5-TCP concentration = 0.46 – 5.15 mg/L; pH 6-8). The solution of these equations using the Euler method for numerical integration of a system of ordinary

differential equations in combination with a conservation of mass equation provided a range of combinations of C_i and C_p values with time for each data set. C_i values for each k_p value were then paired with the explicitly-measured β values for each corresponding experimental time point. β was expressed as fractional inhibition, *i.e.,* 1-β, to produce a series of dose-response curves for each k_p tested, using fractional inhibition (1-β) as biological response and C_i as dose. Several traditional statistical methods for determination of toxicological parameters derived from dose-response relationships include sigmoidal, logistical, and normal non-linear regression curve fitting, as well as Thompson's method of moving averages for the EC_{50}, and log-probit regression for determination of dose-response relationships (Gad, 1999; Calabrese, 1994; Hayes, 1994; Govindarajulu, 1988; Rand, 1995). For k_p values over 0.05 hr^{-1}, data followed a sigmoidal relationship between fractional inhibition (1-β) and C_i, therefore non-linear regression was used to fit a 3-parameter sigmoidal curve to these experimental data to formulate a dose-response relationship for each k_p value tested (Equation 3).

$$\beta = 1 - \frac{a}{1 + e^{-\left(\frac{\frac{C_i}{P} - CPR_{50}}{b}\right)}} \qquad (3)$$

In Equation 3, P (g) is mass of plant and a and b are curve-fitting parameters. CPR_{50} (mg/g) is critical plant residue at which plants are 50% inhibited i.e., mass of internal unprocessed 2,4,5-TCP per g of plant at which plants are 50% inhibited. The CPR_{50} term was used inline with the concept of critical body residue (CPR) which is defined as the whole-body concentration of a chemical that is associated with a given adverse biological response (Rand, 1995). Parameters found for sigmoidal fits with each k_p value are given

in Table 7.1. Figure 7.10 gives an example sigmoidal fit to a dose-response relationship between 1-β and C_i for $k_p = 0.1\ hr^{-1}$.

Measured contaminant removal profiles from independently-gathered data sets were then used to determine the appropriate k_p value. Predictions of contaminant uptake profiles using initial conditions from each data set were developed for each k_p - sigmoidal toxicity curve combination. Predictions were compared with the corresponding measured data by calculating the sum of the squares of the residuals between predicted and measured aqueous phase 2,4,5-TCP concentration. A comparison of the average of the sum of the squares of the residuals for many k_p values tested is presented in Table 7.1. A model value of $k_p = 0.1\ hr^{-1}$ was selected because it gave the smallest average sum of the squares of the residuals. Values for sums of squares of residuals for low k_p values ($k_p = 0.08 – 0.1 hr^{-1}$) were similar to one another indicating that modeling techniques used were not very sensitive to the k_p parameter.

Comparison of Contaminant Uptake and Enzymatic Processing Predictions with Independent Data.

Prediction of contaminant uptake and enzymatic processing were explored by applying the conceptual model outlined in Figure 7.7 and Equations 1 and 2 to independently gathered data sets which had a variety of initial contaminant concentrations, media pH values and plant activities. Contaminant uptake was examined using the previously defined Freundlich isotherm (Figure 6.4), κ relationship ($\kappa = 1.48 + 5.15f$; Chapter 6) and dose-response curve shown in Figure 7.10 for the k_p value of $0.1 hr^{-1}$. Initial

Table 7.1. Parameters for dose-response relationship and sum of square of residual between measured and predicted contaminant mass.

k_p (hr^{-1})	Non-Linear Regression Parameters for Equation 3				Average SSR
	CPR_{50} (mg/g)	a	b	R^2	
0.08	0.115	1.22	0.045	0.63	0.00603
0.10	0.104	1.27	0.039	0.68	0.00601
0.15	0.077	1.22	0.028	0.72	0.00642
0.20	0.062	1.19	0.024	0.70	0.00683
0.25	0.054	1.17	0.022	0.66	0.00712
0.30	0.047	1.15	0.021	0.61	0.00655
0.40	0.035	1.05	0.017	0.52	0.00697

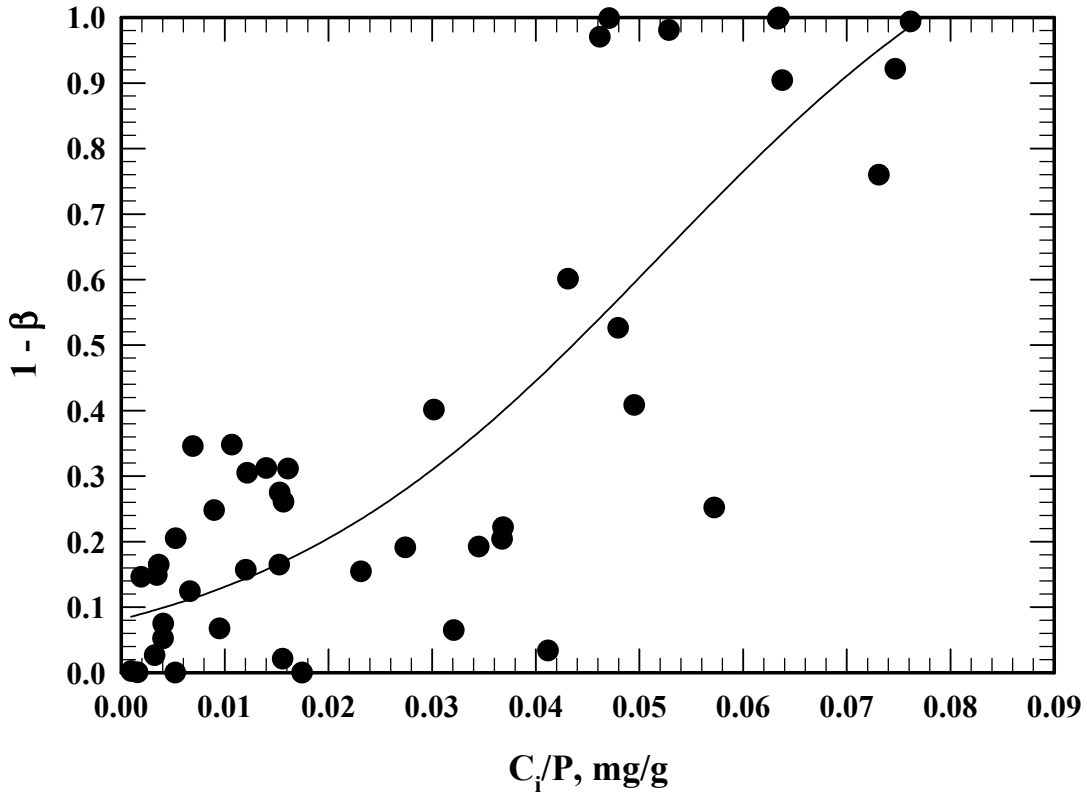


Figure 7.10. Dose-response relationship is shown for predicted C_i/P values and measured fractional activity, 1-β, for $k_p = 0.1\ hr^{-1}$.

contaminant concentrations, initial plant activity values and media pH values for experimental systems shown were used in the conceptual model outlined herein to generate model predictions of contaminant uptake and product accumulation internal to plants. Model predictions are compared with measured aqueous concentration data in Figures 7.11 - 7.13 with predictions of relative plant shown on the second y-axis for plant systems that were uninhibited, partially inhibited and fully inhibited. Data in Figures 7.11 and 7.12 were gathered from reactors where 2 g of plants were exposed to an initial concentration of 5.7 mg/L, α = 0.020 mmol/hr, in 120 mL of media with pH values of 6 and 8, respectively. Data in Figure 7.13 represents 1 g plant exposed to 1.55 mg/L of 2,4,5-TCP in 100 mL media at a pH value of 5 (parameters used were modifies to reflect appropriate plant concentrations). Aqueous phase concentration predictions followed the uptake patterns observed in experimental systems.

Data presented in Figure 7.11 represent plants exposed to a relatively high concentration of 2,4,5-TCP (5.7 mg/L) at a low pH value (pH = 6). Aqueous 2,4,5-TCP data showed that uptake was initially rapid, followed by aqueous phase 2,4,5-TCP concentrations reaching a plateau value. Rapid initial uptake of 2,4,5-TCP meant that a large mass of 2,4,5-TCP quickly accumulated internal to plants. Modeled values of 2,4,5-TCP internal to plants (C_i) rapidly accumulated at initial times and a plateau value of C_i was reached at times which corresponded to aqueous concentration plateaus (Figure 7.11). Enzymatic processing of 2,4,5-TCP occurred at initial time points, but ceased after ~10 hr as indicated by the relatively small accumulation of processed 2,4,5-TCP internal to plants (C_p). Projections of relative plant activity (Figure 7.11) showed that toxic effects began

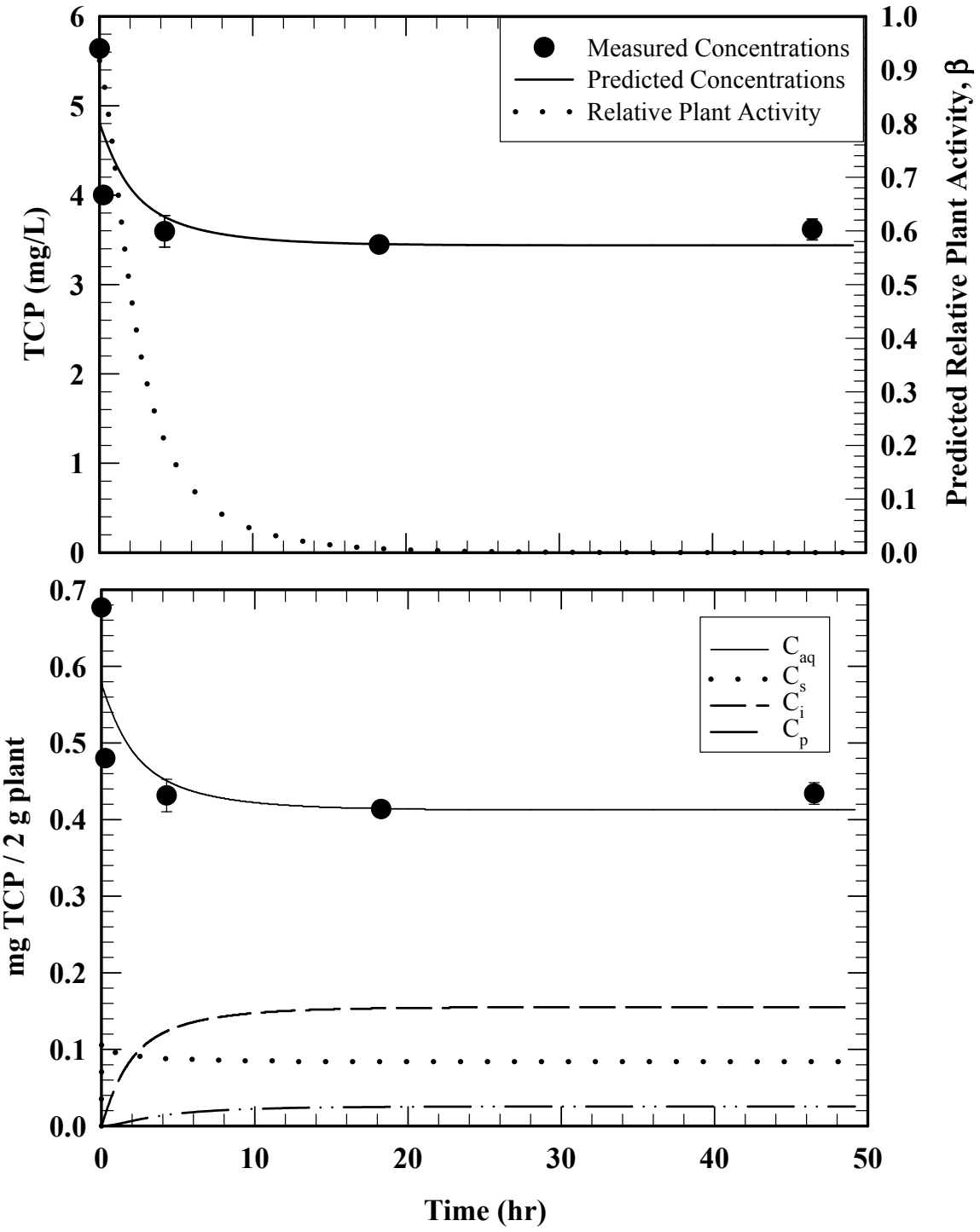


Figure 7.11. Model predictions for independent data (5.7 mg/L 2,4,5-TCP; pH = 6) are compared with measured concentration profile. Predicted relative plant activity data are shown on the second y-axis of the top figure. Predicted profiles of C_s, C_i and C_p are presented on the lower graph.

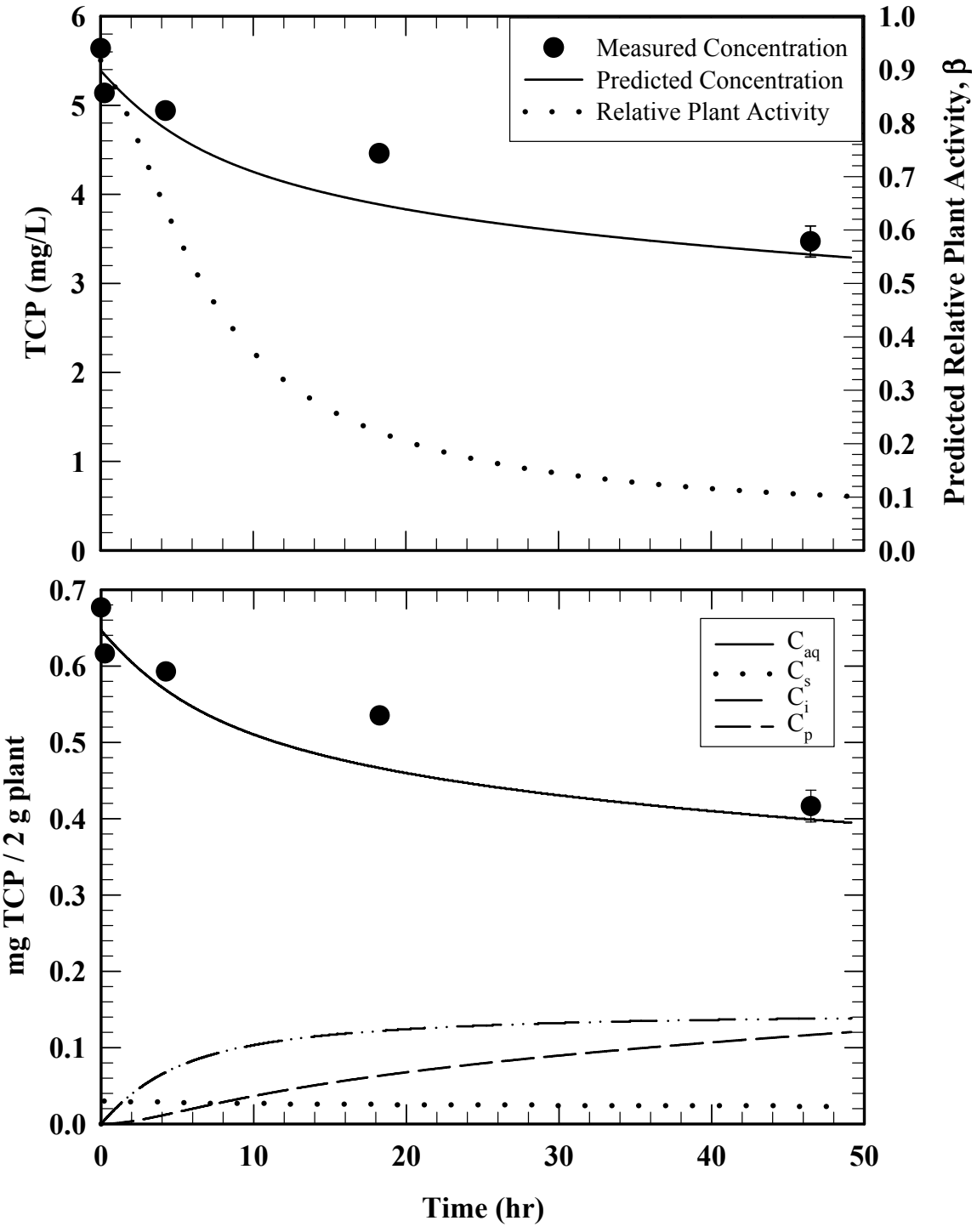


Figure 7.12. Model predictions for independent data (5.7 mg/L 2,4,5-TCP; pH = 8) are compared with measured concentration profile. Predicted relative plant activity data are shown on the second y-axis of the top figure. Predicted profiles of C_s, C_i and C_p are presented on the lower graph.

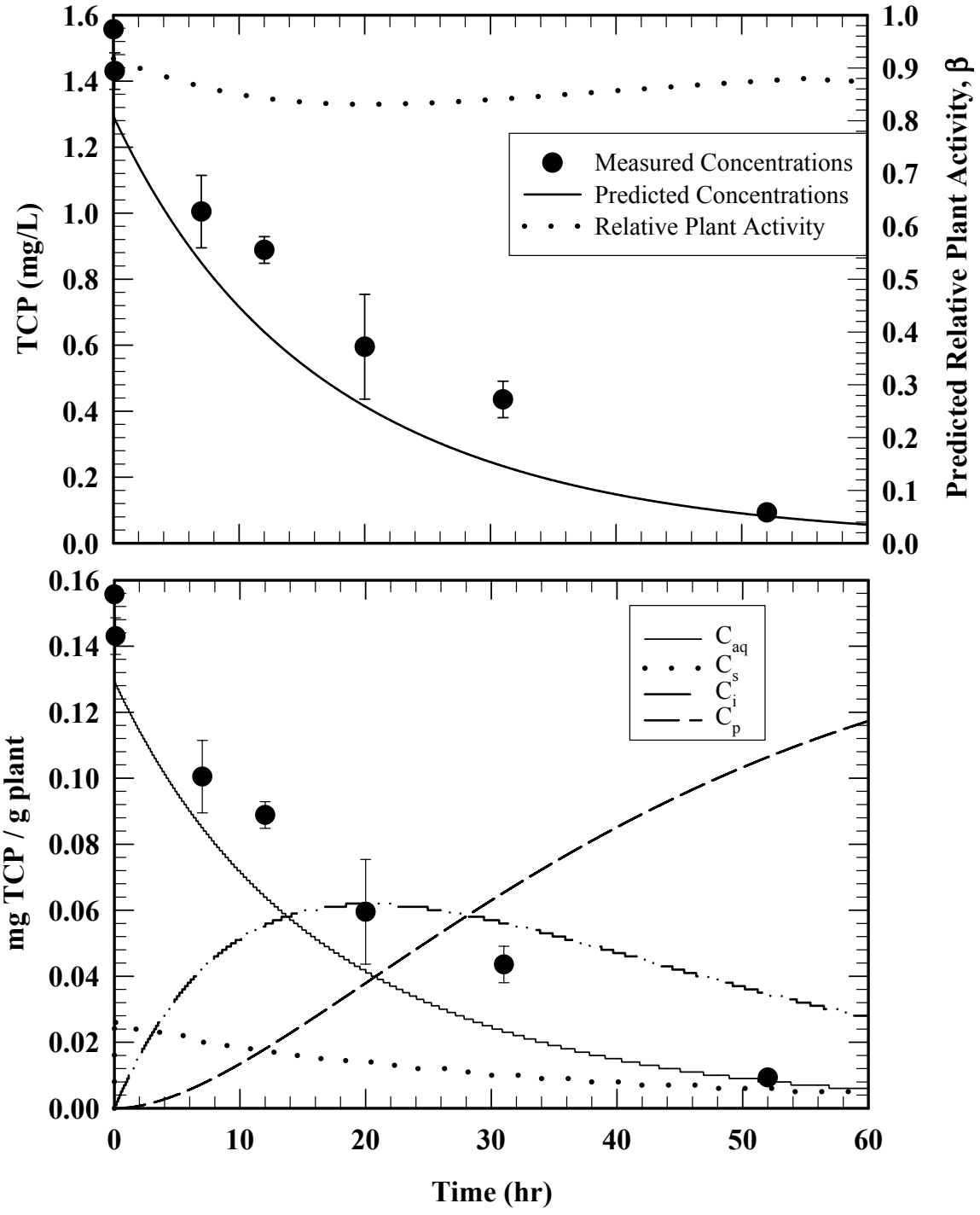


Figure 7.13. Model predictions for independent data (1.55 mg/L 2,4,5-TCP; pH = 6) are compared with measured concentration profile. Predicted relative plant activity data are shown on the second y-axis of the top figure. Predicted profiles of C_s, C_i and C_p are presented on the lower graph.

almost immediately, where relative plant activity decreased rapidly and reached a value near zero after only ~10 hr. Contaminant uptake and enzymatic processing were dependant on relative plant activity, therefore, zero values for relative plant activity indicated no further enzymatic processing of contaminants or uptake by plants occurred.

The projection of plateau concentrations of C_{aq}, C_i and C_p paralleled the decrease and zero value of relative plant activity projected. The plateau concentration of projected C_{aq} was consistent with the plateau concentration observed in aqueous concentration measurements. The close fit of model predictions to measured data demonstrated the ability of the conceptual model to predict trends in uptake rate which depended on inhibition.

Data presented in Figure 7.12 represent plants exposed at pH 8 to the same high concentration of 2,4,5-TCP (5.7 mg/L) as those shown in Figure 7.11. Aqueous phase 2,4,5-TCP measurements indicated that uptake proceeded more slowly in the pH 8 system than in the pH 6 system, and model predictions paralleled the decrease in uptake rate. Projected mass of contaminant internal to plants (C_i) accumulated more slowly in pH 8 systems (Figure 7.12) and accumulation of C_p occurred throughout the experiment. Relative plant activity decreased slowly in the pH 8 system when compared with the pH 6 system due to the more gradual accumulation of 2,4,5-TCP internal to plants (Figure 7.12). Aqueous phase concentration measurements were closely paralleled by modeled aqueous phase concentrations, demonstrating that the conceptual model can be used to predict contaminant concentrations in partially inhibited systems.

Model predictions presented in Figure 7.13 were for 1.5 mg/L 2,4,5-TCP with a media pH of 5. Contaminant uptake proceeded rapidly with first-order kinetics, and projected values closely matched measured concentrations (Figure 7.13). Projected relative plant activity indicated that inhibition did not occur, a finding which was consistent with the rapid first-order uptake. 2,4,5-TCP internal to plants (C_i) was projected to increase rapidly at initial times, and slowly decrease at later time points. Enzymatically processed contaminant (C_p) was projected to increase throughout the experiment shown. Projections of C_i and C_p were consistent with rapid partitioning of 2,4,5-TCP into plants as expected at low pH values in parallel with continuing enzymatic processing of contaminants expected when inhibition was not observed.

Model predictions presented in Figure 7.14 – 7.15 were for 2.2 mg/L 2,4,5-TCP, $\alpha = 20.3$ μmol/hr with media pH values of 6 and 8, respectively. In the pH 6 system (Figure 7.14) contaminant uptake proceeded rapidly at initial times, slowing slightly at later times in the experiment. Prediction of aqueous phase concentrations matched the uptake profile at initial time points, however, rate of uptake was over predicted at later time points. Projected relative plant activity values decreased until a value of $\beta < 0.5$ was reached after ~15 hr, after which projected relative plant activity increased until a value $\beta = \sim 1$ was reached at the end of the experiment. In the pH 8 system (Figure 7.15), contaminant uptake proceeded at a slow, first-order rate throughout the experiment. Predictions provided by the conceptual model overestimated rate of uptake from aqueous phase, but the continuous uptake and minimal inhibition observed were consistent with measured concentrations.

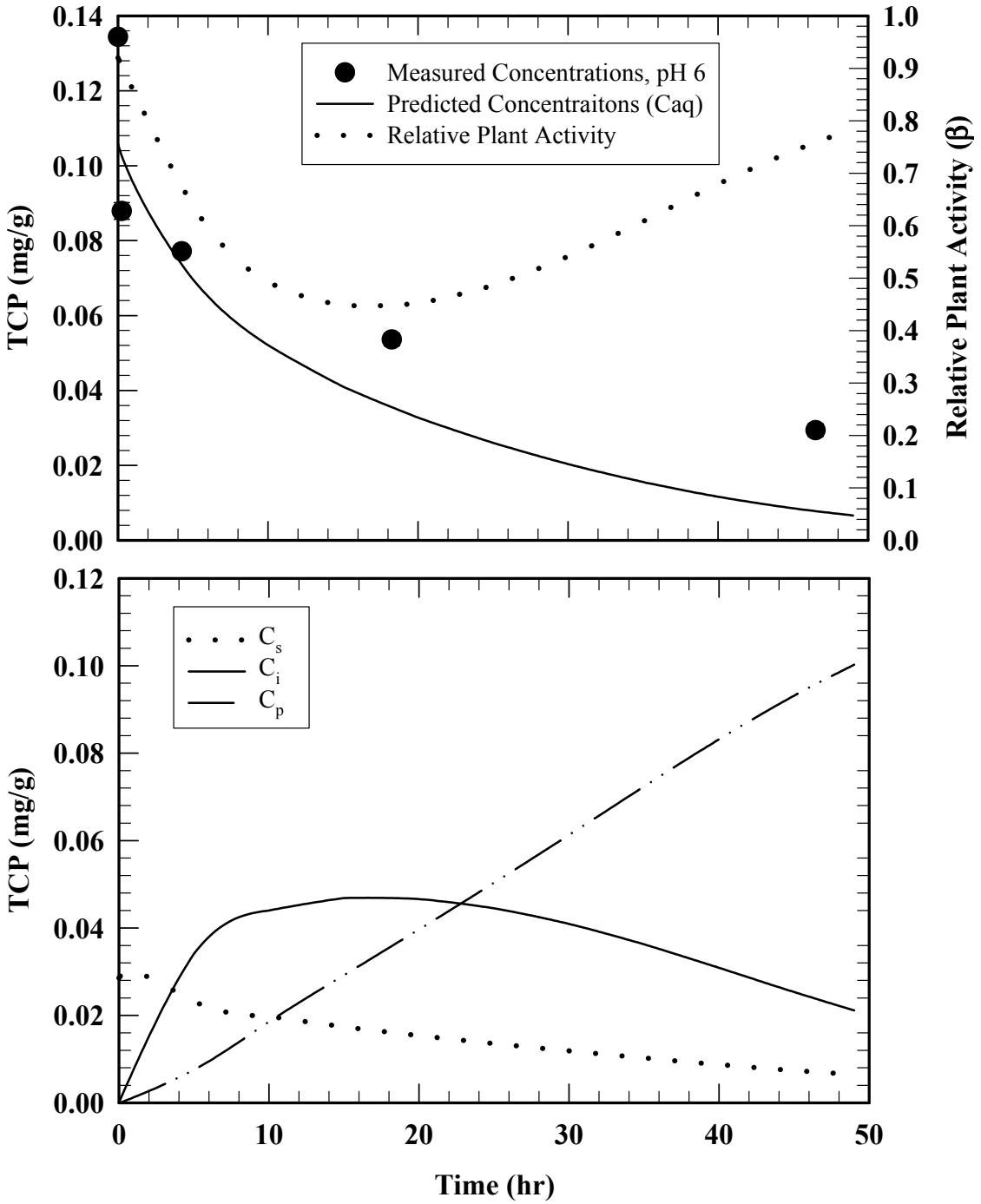


Figure 7.14. Model predictions for independent data (2.2 mg/L 2,4,5-TCP; pH = 6) are compared with measured concentration profile. Predicted relative plant activity data are shown on the second y-axis of the top figure. Predicted profiles of C_s, C_i and C_p are presented on the lower graph.

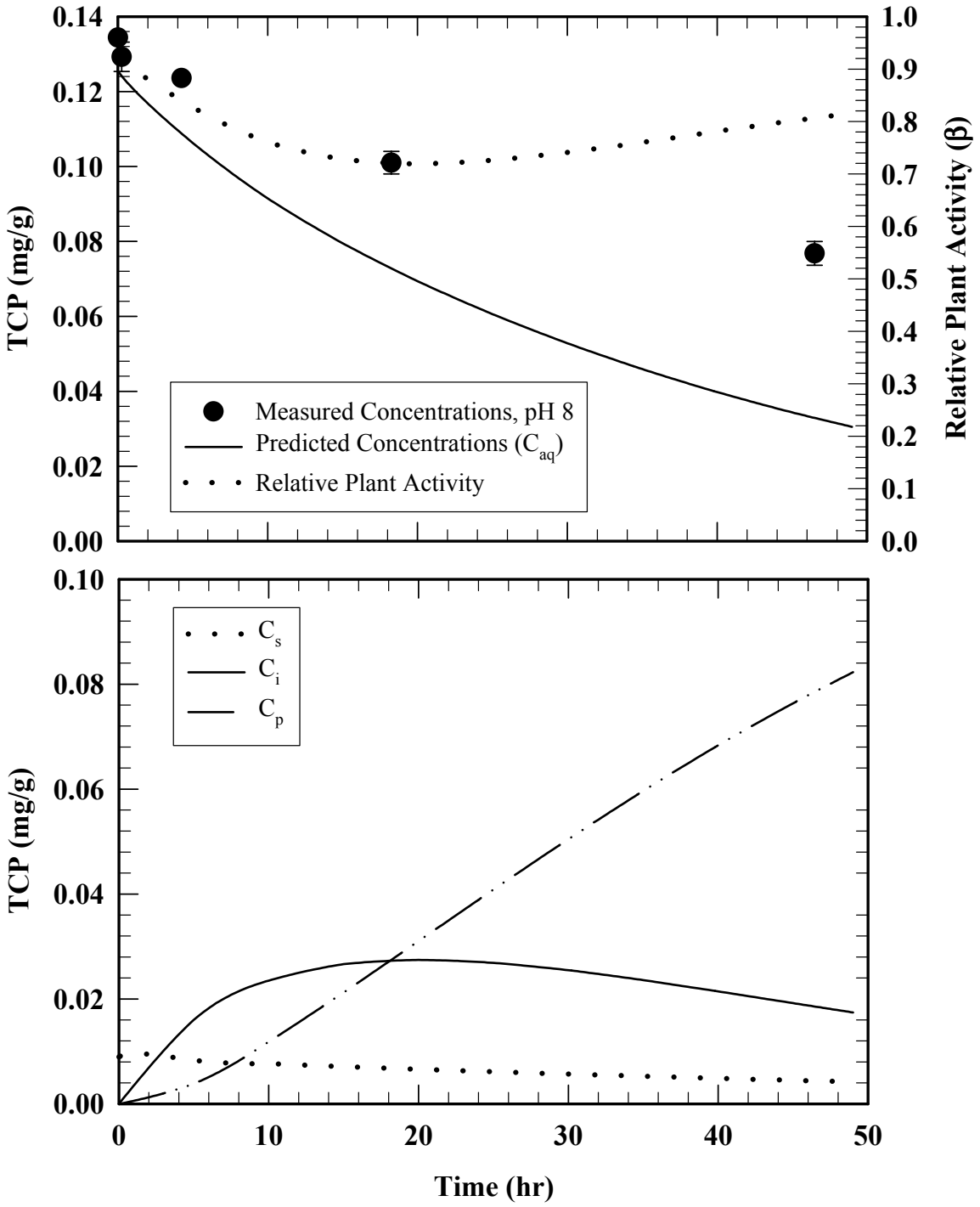


Figure 7.15. Model predictions for independent data (2.2 mg/L 2,4,5-TCP; pH = 8) are compared with measured concentration profile. Predicted relative plant activity data are shown on the second y-axis of the top figure. Predicted profiles of C_s, C_i and C_p are presented on the lower graph.

The capacity of the conceptual model to describe mass of internal 2,4,5-TCP and enzymatically processed 2,4,5-TCP was assessed by applying the conceptual model to 2,4,5-TCP uptake and processing data for *L. minor* presented by Day (2002). A data set presented by Day (2002) outlined 2,4,5-TCP uptake from aqueous phase, accumulation 2,4,5-TCP internal to plants (C_i), and accumulation of 2,4,5-trichlorophenyl-β-D-glucopyranoside (TCPG), 2,4,5-trichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside (TCPMG) and 2,4,5-trichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuanoside (TCPAG) internal to plants. The distribution of measured 2,4,5-TCP components internal to plants is detailed in Table 7.2 for an experiment which consisted of reactors contained 100 mL media, 3 g plant and an initial concentration of 16.2 μmol/L (i.e., 3.2 mg/L) where media pH, plant activity and relative plant activity were not measured. Measured aqueous concentration values demonstrated a rapid initial drop in concentration, followed by uptake which proceeded at a first-order rate. Accumulation of 2,4,5-TCP internal to plants (C_i) showed a rapid accumulation of C_i at initial times, followed by a slow decrease in C_i at later times. Product formation showed a steady concentration of TCPG throughout the experiment, rapid initial accumulation of TCPMG and steady accumulation of TCPAG throughout the experiment. Quantities of total transformation products (C_p) increased throughout the experiment, where the rate of increase of C_p was more rapid at initial times than later time points. The dataset presented in Table 7.2 achieved only partial mass balance closure (77 – 85%) with decreasing mass recoveries at later time points.

Table 7.2. Data for uptake and enzymatic processing of 2,4,5-TCP by *L. minor* taken from Day (2002).

Time (hr)	C_{aq} (μmol)	C_i (μmol)	TCPG (μmol)	TCPMG (μmol)	TCPAG (μmol)	C_p* Measured	% Recovered	C_p * Difference
0	1.62	0	0	0	0	0	100	0
22	0.44	0.36	0.09	0.46	0.03	0.58	85	0.82
48	0.26	0.2	0.05	0.71	0.09	0.85	81	1.16
70	0.12	0.15	0.08	0.75	0.17	1.00	78	1.35
94	0.04	0.1	0.04	0.86	0.25	1.15	80	1.48
120	0.02	0.08	0.06	0.81	0.27	1.14	77	1.52

*$C_{p,\ measured}$ = TCPG + TCPMG + TCPAG; $C_{p,\ difference} = C_T - (C_{aq}+C_i)$; C

The conceptual model was applied to the data set provided by Day (2002) presented in Table 7.2. High levels of removal were noted at the initial time points indicating that significant levels of 2,4,5-TCP sorbed to plant surfaces. Therefore, an assumed media pH of 6 was used. Model predictions of C_{aq} were fit to measured aqueous phase concentrations for the data set detailed in Table 7.2 by varying plant activity to achieve a minimum sum of squares of the residuals. The value of α produced from fitting C_{aq} was $\alpha = 0.0089$ mmol/hr which was in the range of α values observed in experimentation detailed herein. The fitted C_{aq} projection is shown with measured data in Figure 7.16, demonstrating the close fit achieved by modifying α to project contaminant uptake.

The conceptual model was employed using the parameters which best fit the rate of 2,4,5-TCP uptake by plants (pH = 6, $\alpha = 0.0089$ mmol/hr) and values of C_i and C_p were predicted using the conceptual model. Predictions of contaminant mass distribution indicated rapid increase in C_i, followed by a slow decline in C_i until little C_i was observed at later time points. Predicted values of C_i are compared with measured 2,4,5-TCP internal to plants in Figure 7.16 and predicted values closely matched measured values provided by Day (2002) demonstrating ability of the model to appropriately project fluctuations of 2,4,5-TCP internal to plants. No inhibition was projected by the conceptual model, as is shown by relative plant activity values $\beta \sim 1$ in Figure 7.16. The projection of no inhibition was consistent with maximum measured and predicted C_i values (< 0.17 μmol/g) which were significantly lower than CPR_{50} value of 0.53 μmol/g. Prediction of enzymatically processed contaminant (C_p) is shown in Figure 7.16. C_p rapidly increased during initial times and continued to increase through the end of the

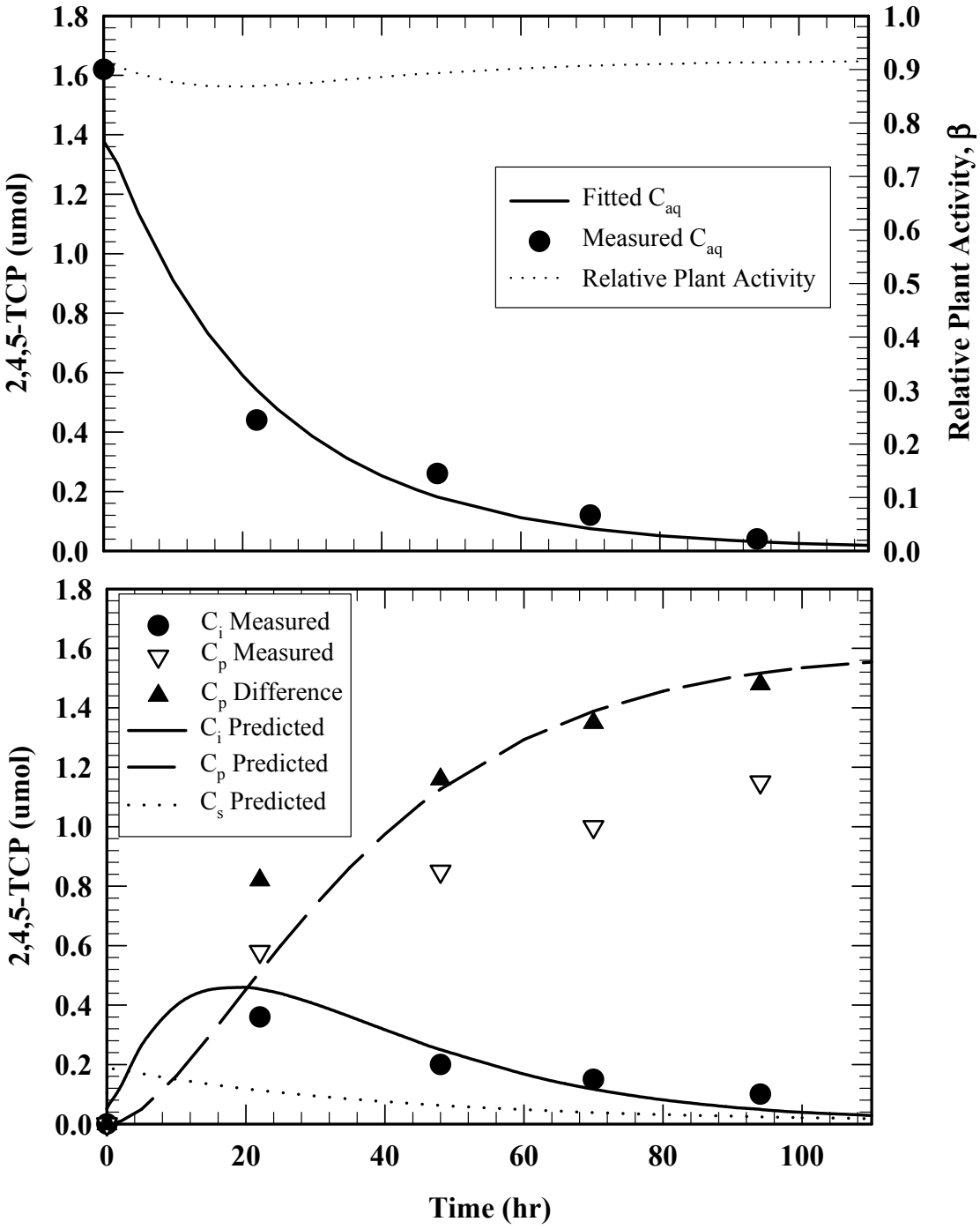


Figure 7.16. Model predictions are compared with independent data provided by Day (2002) where C_{aq}, C_i, and C_p were measured. *L. minor* was exposed to 3.2 mg/L 2,4,5-TCP and an assumed pH value of 6 was used due to high levels of sorption observed. Predicted relative plant activity data are shown on the second y-axis of the top figure. Predicted profiles of C_s, C_i and C_p are presented on the lower graph.

experiment, although values increased less rapidly at later times when C_i and C_{aq} were low. Predicted C_p values were compared with the sum of measured products of TCP (i.e., $C_{p,\ measured}$ = TCPG + TCPMG + TCPAG). Predicted values of C_p followed the same trend as $C_{p,\ measured}$ (rapid initial increase, less rapid but continued increase at later times) however, the model over predicted C_p when compared with values of $C_{p,\ measured}$. The data set presented in Table 7.2 from Day (2002) achieved only partial mass balance closure (77% – 85%), with decreasing mass balance closure at later time points. To investigate the over prediction of C_p when compared with $C_{p,\ measured}$, predictions of C_p were compared with mass of contaminant that was not detected as C_i or C_{aq} ($C_{p,\ difference}$ = $C_T - (C_{aq} + C_i)$) where C_T was the total mass of contaminant in the system and therefore complete mass balance closure was an inherent assumption. Predicted values of C_p closely matched $C_{p,\ difference}$ (Figure 7.16). Appropriate prediction of the trend for C_i and C_p values for the independent data set presented by Day (2002) using the conceptual model demonstrated the ability the conceptual model to describe internal accumulation and processing of TCP by plants.

The conceptual model was further compared with an additional data set provided by Day (2002) which measured the readily extractable fraction of TCP internal to plants (free-TCP) and the fraction of TCP which was extractable when treated with moderate heat and strong-acid (product-bound-TCP). Experimental parameters were an initial concentration of TCP of 3.32 mg/L TCP, 100 mL media and 2 g plant where pH and plant activity were not measured. Free-TCP was considered to be the TCP internal to plants (the equivalent of C_i) and was shown to increase at initial times, but was not detected after 48 hr of

exposure. Product-bound-TCP was considered to be similar to $C_{p,measured}$ and rapidly increased throughout the experiment. Data for free-TCP and product-bound-TCP are shown in Table 7.3, along with mass recovery data.

The conceptual model was applied to the data presented in Table 7.3, where a pH value of 6.9 was fitted using the 2 hr data point and the Freundlich isotherm previously presented (Chapter 4). Least squares of residuals was used to fit an α value to the dataset used by comparing measured aqueous phase concentrations and modeled C_{aq} (α = 4.1 μmol/hr). The conceptual model was employed using the parameters which best fit the rate of TCP uptake by plants (pH = 6.9, α = 4.1 μmol/hr) and values of C_i and C_p were predicted. Predictions of C_i demonstrated a moderate increase in C_i and a plateau value was reached after ~20 hr (Figure 7.17). Predictions of C_i matched the initial uptake profile when compared with measured free-TCP, however predictions of C_i over predicted free-TCP internal to plants at later times (i.e., 24 and 48 hr). Prediction of C_p indicated linear accumulation of enzymatically processed contaminant throughout the experiment. Quantity of C_p for time points of 2 hr and 48 hr was over predicted using the conceptual model when compared with measured values of product-bound-TCP.

Discussion

Quantitative relationships have been established for (i) inhibition of aquatic plant bioprocesses by parent contaminant internal to plants and (ii) contaminant uptake by aquatic plants as a function of plant metabolic activity, pH and inhibition. Inhibition of plants by contaminants was demonstrated through a comparison of oxygen production

Table 7.3. Data for uptake and enzymatic processing of TCP by *L. minor* taken from Day (2002).

Time (hr)	Aqueous Phase TCP (μmol)	Free-TCP (μmol)	Product-bound TCP (μmol)	% Recovered
0	1.7	0	0	100
2	1.5	0.06	0.09	97.1
24	1.1	0.09	0.34	87.1
48	0.9	0.34	0.47	80.5

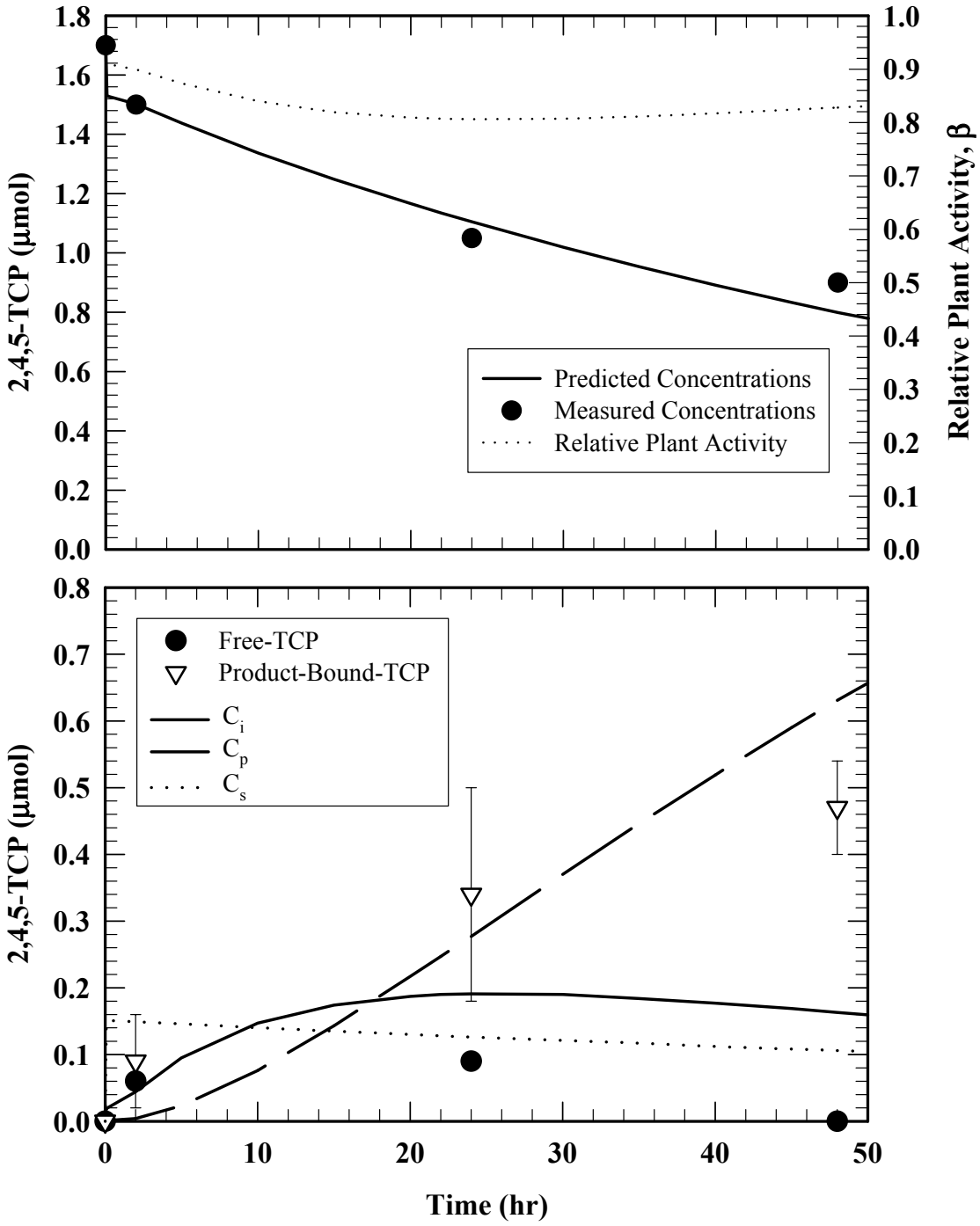


Figure 7.17. Model predictions are compared with independent data provided by Day (2002) where C_{aq}, C_i and C_p were measured. *L. minor* was exposed to 3.3 mg/L 2,4,5-TCP and an fitted pH value of 6.9 was used. Predicted relative plant activity data are shown on the second y-axis of the top figure. Predicted profiles of C_s, C_i and C_p are presented on the lower graph.

measurements in contaminant treated systems with those of control systems. Relative plant activity (β) was the parameter used to gauge inhibitory effects on plant metabolism. β values ranged from one (i.e., oxygen production rate similar to that of control systems and no inhibition) to zero (i.e., no plant production of oxygen and plants were fully inhibited by contaminants). Relative plant activity (β) was compared with the plant bioconcentration factor (PBCF) and a threshold value of approximately 100 μg TCP / g plant, was apparent where toxic effects were observed at PBCF greater than 100 μg TCP / g plant. However, the toxicity threshold value was not absolute and toxicity was observed as PBCF lower than 100 μg TCP / g plant and in some cases no toxicity was observed at PBCF greater than 100 μg TCP / g plant. These results indicated that complex relationships underlay contaminant-driven toxicity.

A conceptual model (Figure 7.7) was established for use in characterization of aquatic-plant assimilation of contaminants in batch applications and it has been validated using independently gathered data sets. This relationship can be used in describing and predicting contaminant fate in the natural environment and phytoremediation processes. These results clearly establish dependence of contaminant uptake and assimilation on photosynthetic activity.

Experimental data demonstrated that toxicity decreased or stopped contaminant uptake by plants. Results showed that when contaminant uptake rate was fast relative to enzymatic processing rate, an excess of unprocessed contaminant accumulated internal to plants and toxic effects due to unprocessed contaminant internal to plants were observed. The

conceptual model accounted for the accumulation of trace-organic contaminants by aquatic plants and incorporated plant inhibition into a description of contaminant uptake. Contaminant uptake was observed to be drastically reduced or stopped in inhibited systems, demonstrating that toxicity has a dramatic impact on system performance and remediation potential confirming the critical need for assessment of toxicity in phytoremediation of contaminants.

Data presented show that toxicity of an ionizable organic contaminant was pH dependant. Coupling relative plant activity data with TCP uptake data indicated that contaminant uptake rate, rather than external contaminant concentrations, dictated toxic effects. Systems with the same initial external concentrations consistently showed significant toxic effects at lower pH values due to accelerated uptake rate that occurred at lower pH values. Toxicity was indirectly shown to be a function of internal mass of contaminant. These data agree with previous reports which demonstrated increased toxicity of ionizable contaminants to several floral and faunal species at low pH values (Neilson *et al.,* 1990; Saarikoski and Viluksela, 1981; Svenson and Zhang, 1995; Blackman *et al.,* 1955a; Blackman *et al.,* 1955b).

In this regard, there has been extensive use of *Lemna* species as a toxicity bioassay and in whole effluent testing (WET). Data quantifying inhibition of *L. minor* by TCP have been reported in the literature. Ensley and co-workers (1997) report the 7 d EC_{50} for frond production as 0.414 (±0.0395) mg/L for 15 fronds and Blackman and co-workers (1955a; 19555b) report a 48 hr LD_{50} of 1.68 mg/L for 50 fronds. These previous toxicity

examinations rely totally on measures of reduced biomass production as the toxic effect endpoint without examining contaminant assimilation or plant process inhibition. Saarikoski and Viluksela (1981) report 96 hr LD_{50} values for *Poecilia reticulate* exposure to TCP as 0.99 mg/L at pH 6 and 2.96 mg/L at pH 8, demonstrating a 3-fold difference in lethal concentration with a 2-unit pH change. Results for pH dependant toxicity of *P. reticulate* are consistent with data presented herein and demonstrate the likelihood of incorrectly assessing toxicity when contaminant assimilation is not specifically considered.

The research presented herein introduced the CPR_{50} term in Equation 3 as mass of internal unprocessed TCP at which plants are 50% inhibited per g of plant. The CPR_{50} term is consistent with the lethal body burden and critical body residue (CPR) concepts used in aquatic toxicology where mortality is associated with contaminant residue levels in the body (Rand, 1995). Rand (1995) stated that advantages of using CPR over surrogates based on exposure media include the fact that CPR explicitly includes accumulation kinetics and effects of metabolism on accumulation and that bioavailability is explicitly considered in CPR. CPR_{50} quantifies mass of 2,4,5-TCP actually causing toxic effects in plants therefore external factors such as plant activity, pH and total plant mass do not skew results.

The CPR_{50} value found for 2,4,5-TCP in this model was 0.104 mg/g plant based on data collected for a range of pH values, plant activity values and 2,4,5-TCP concentrations. CPR_{50} is fundamentally different from traditional toxicity assessment methods (i.e., EC_{50}

and LD_{50}) and cannot be directly compared with values reported from traditional toxicity assessments. CPR_{50} considers the contaminant mass that has partitioned into plants and rate of contaminant detoxification by plants, whereas traditional methods of toxicity assessment consider only external concentration.

Consideration of CPR_{50} values and modeling results outlining increased toxicity caused by faster rates of contaminant partitioning into plants lends validity to previous reports of decreased toxicity with increasing plant mass (Wang and Freemark, 1995; Ensley *et al.,* 1997). If plant mass is decreased, the mass of contaminant available to partition into each plant is greater, therefore levels of unprocessed contaminant internal to the plant (C_i) will be increased in lower plant mass systems. CPR_{50} accounts for mass of unprocessed contaminant internal to plants per mass of plant, thus allowing for comparison between systems with varying plants masses.

Our studies have shown that *L. minor* served as an excellent model plant for examining organic-contaminant assimilation by plants, as well as the associated inhibitory effects and contaminant fate in plants. This work builds on the base of knowledge developed through use of *Lemna* spp. in toxicity bioassays and WET, and provides an enhanced understanding of plant-contaminant interactions for use in such assays. However, results shown herein demonstrate the need to examine conditions of exposure more thoroughly because contaminant uptake rate is driven by environmental factors such as pH and plant activity. For example, in WET, toxicity is assessed for complex effluents without regard for factors such as pH values of effluents and receiving streams. The studies presented

herein demonstrate that pH is a critical element for contaminant uptake and toxic effects, and results could be potentially skewed by disregarding this critical parameter. Additionally, comparison between toxicity studies is impossible when plant activity is not assessed for individual systems because plant activity is an underlying factor in contaminant uptake and therefore toxicity. Results presented herein have demonstrated the importance of considering factors such as pH and plant activity in assessing toxicity.

CHAPTER 8

ANAEROBIC MICROBIAL DEGRADATION OF PLANT SEQUESTERED CONTAMINANT

A biological assay was used to assess fate of plant-sequestered chlorinated phenols. After plant death, plant detritus typically moves into sediment dominated by anaerobic microbial activity. The reducing conditions are derived from the decomposition of plant biomass. Anaerobic reactors were used to explore the fate of plant-sequestered chlorinated phenols associated with anaerobic sediments.

Desulfitobacterium sp. strain Viet1, the primary microorganism used in the bioassay detailed herein was obtained from the laboratory of Dr. Frank Löffler at the Georgia Institute of Technology. This work was a collaborative effort with the Löffler research group and doctoral candidate Benjamin Amos.

Reduction of halogenated phenols by *Desulfitobacterium* sp. strain Viet1

The chlororespiring organism, *Desulfitobacterium* sp. strain Viet1, has been shown by Löffler (2003) to utilize ortho-positioned chlorophenols as terminal electron acceptors (e.g., 2,4-DCP, 2,4,6-TCP, and 2-CP). Isolate Viet1 can reduce the phenol ring at the ortho-positioned chlorine, while the para- and meta-positions were not reduced by this

organism. Results provided by Chang (2000) demonstrated that plant-detritus and humic materials adequately serve as the sole electron-donor source for anaerobic reductive dechlorination. Therefore, activity of strain Viet1 was assessed for use in examination of bioavailability and biodegradation of plant-sequestered contaminants.

The activity of strain Viet1 with 2,4-DCP was examined in reduced, plant-free, mineral salts media at concentrations of 30 and 60 μM. Triplicate cultures were incubated in 160 ml bottles containing 100 mL of media and were inoculated with 2% v/v of an actively growing stock culture. Lactate (5 mM) was used as the electron donor and carbon source. No production of 4-CP and no significant change in 2,4-DCP concentration were observed after 3 d in 30 μM reactors (Figure 8.1). In 60 μM reactors, a slight decrease in 2,4-DCP was observed after 3 d and trace quantities of 4-CP were produced (Figure 8.2). After 10 d, 2,4-DCP concentrations dropped significantly in all reactors. In parallel, significant quantities of 4-CP had been produced by 10 d. Small increases in 4-CP concentrations were observed between 10 d and 14 d, and a small mass of 2,4-DCP was depleted in the same time frame. Trace quantities of 2,4-DCP remained in the media at 14 d (< 1 μM). Total mass recovery was calculated as the sum of molar concentrations of chlorophenols (i.e., sum of molar concentrations = [2,4-DCP] + [4-CP]) normalized to total molar concentration of chlorophenol expected in aqueous phase if all contaminant was in the media (i.e., total molar concentration = total 2,4-DCP added / volume). Total mass recoveries were 78.5 (± 3.1) % and 84.4 (± 4.8) % for 30 μM and 60 μM reactors, respectively. Parallel sequences of chlorophenol degradation were observed in 30 μM and 60 μM reactors; therefore strain Viet1 was considered to be uninhibited by 60 μM of

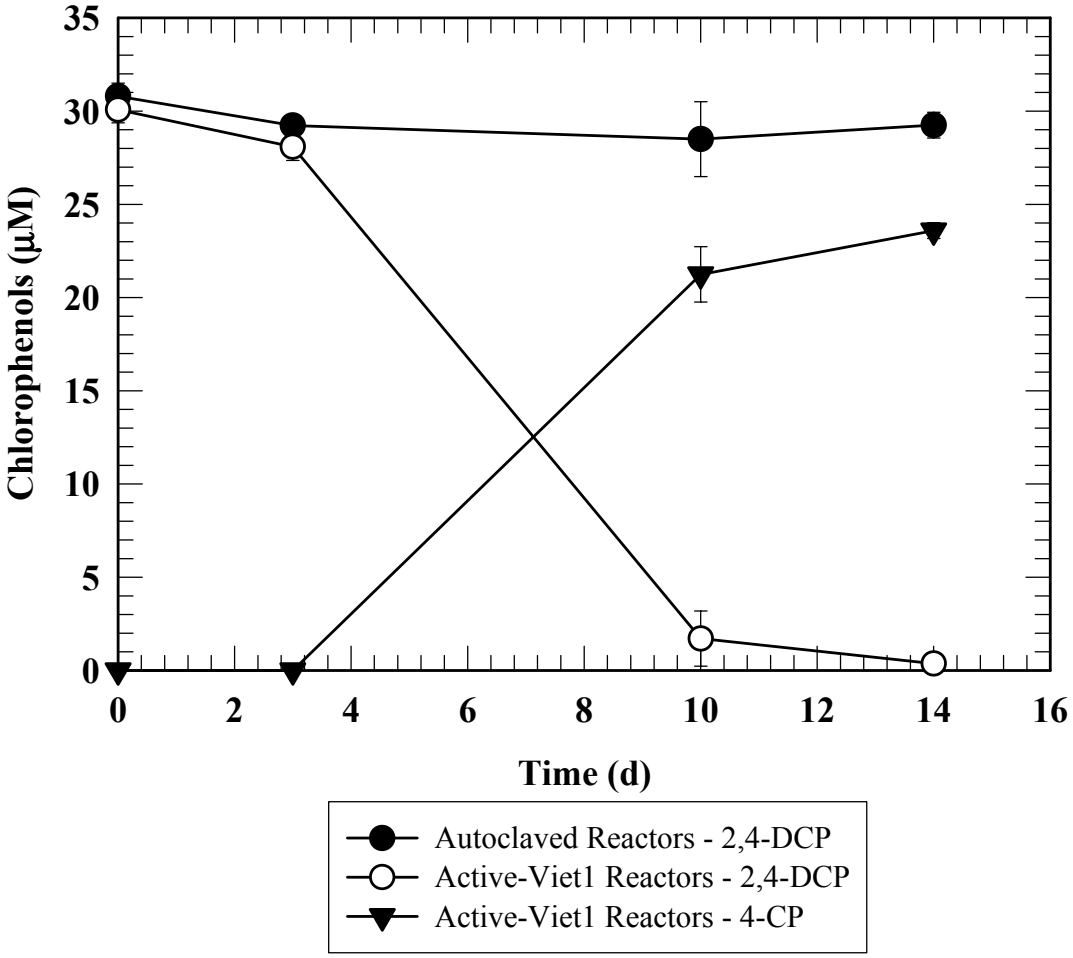


Figure 8.1. Degradation of 30 µM 2,4-DCP by strain Viet1 in plant-free media. No degradation observed in autoclaved controls.

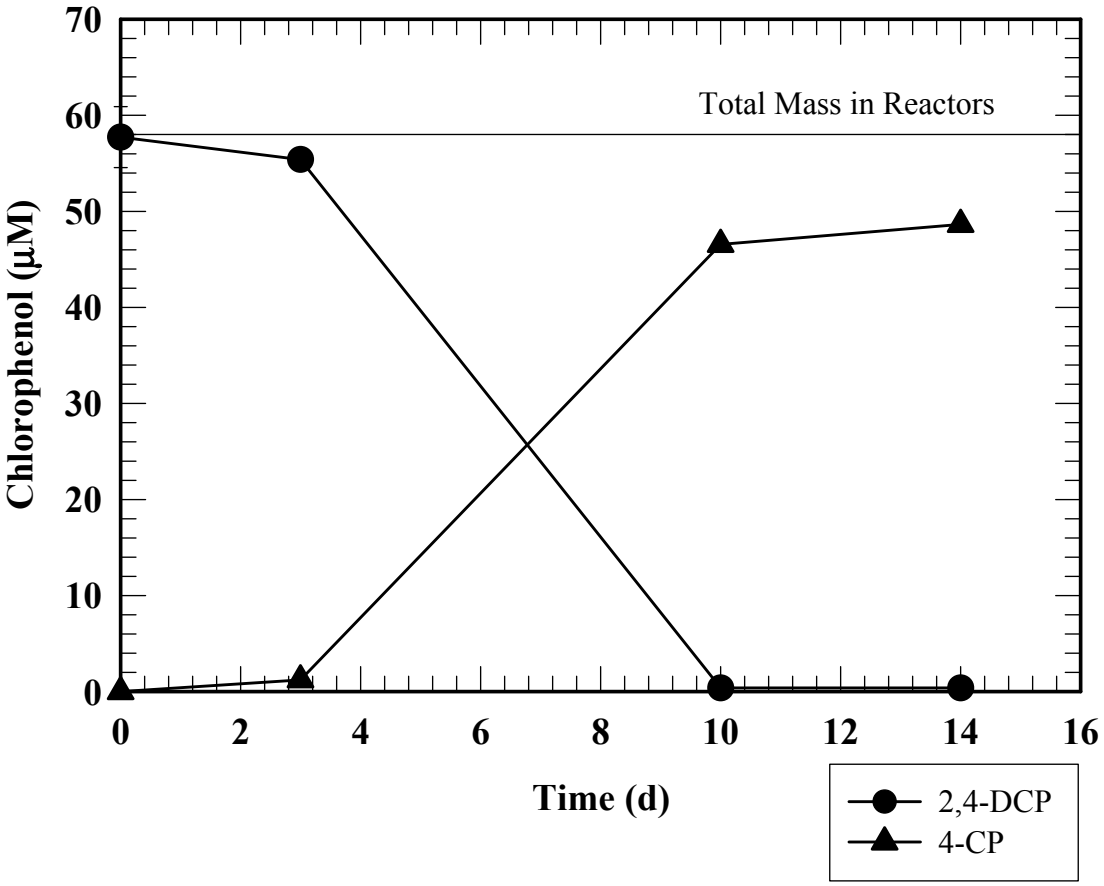


Figure 8.2. Degradation of 60 µM 2,4-DCP by strain Viet1 in plant-free media. Total mass in reactors is provided for reference.

2,4-DCP. Constant 2,4-DCP concentrations were observed in triplicate autoclaved control reactors with media concentrations of 30 μM and no production of 4-CP was observed in autoclaved controls (Figure 8.1). Autoclaved control reactors verified that all dechlorination in isolate Viet1 amended reactors was a result of biological processes.

An additional assay was used to examine the reduction of chlorinated phenols and dechlorination endpoints. Cultures were incubated in 160 ml bottles containing 100 mL of media and were inoculated with 2% v/v of an actively growing stock culture. Lactate (5 mM) was used as the electron donor and carbon source. Degradation of 2,4-DCP and 2,4,6-TCP was examined at concentrations of 26.4 and 25.7 μM, respectively. Results for degradation of 2,4-DCP demonstrated that reduction of the ortho-positioned chlorine had begun by 2 d, and all 2,4-DCP was reduced by 8 d (Figure 8.3). 4-CP was produced in significant quantity by 8 d, and concentration was stable over the 32 d experimental time frame (Figure 8.3). Mass recovered as 4-CP after 32 d was 79.1% of 2,4-DCP added to the experimental system. All 2,4,6-TCP was reduced by strain Viet1 after 5 d (Figure 8.4). Significant masses of 2,4-DCP and 4-CP were produced after 5 d where concentrations of 20.2 μM and 6.1 μM were observed, respectively. After 8 d, all 2,4,6-TCP was converted to 4-CP and the concentration of 4-CP was stable over the 32 d experimental time frame (Figure 8.4). Mass recovered as 4-CP after 32 d was 112.1% of 2,4,6-TCP added to the experimental system. These results demonstrated that strain Viet1 rapidly dechlorinated 2,4-DCP forming 4-CP as a stable end-product. Therefore, *Desulfitobacterium* sp. stain Viet1 was used to assay biologically available 2,4-DCP after sequestration in plants.

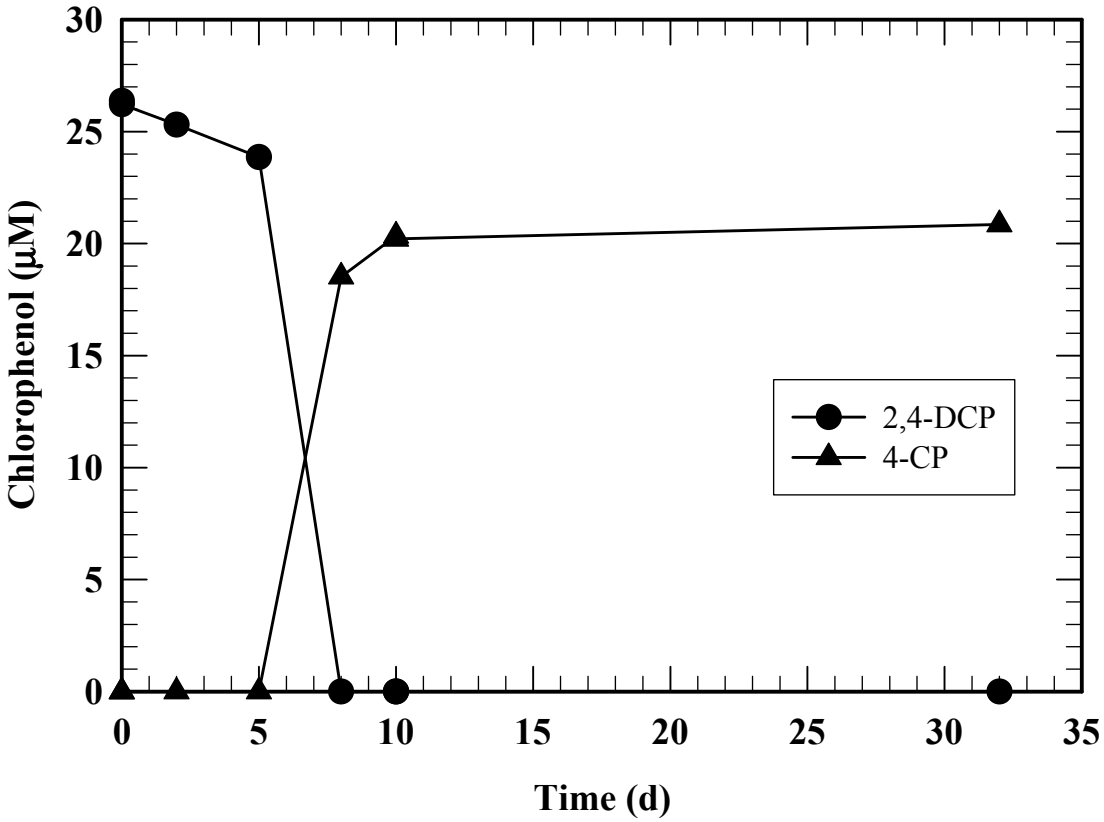


Figure 8.3. Reduction of 2,4-DCP to 4-CP by strain Viet1 over a 32 d time period.

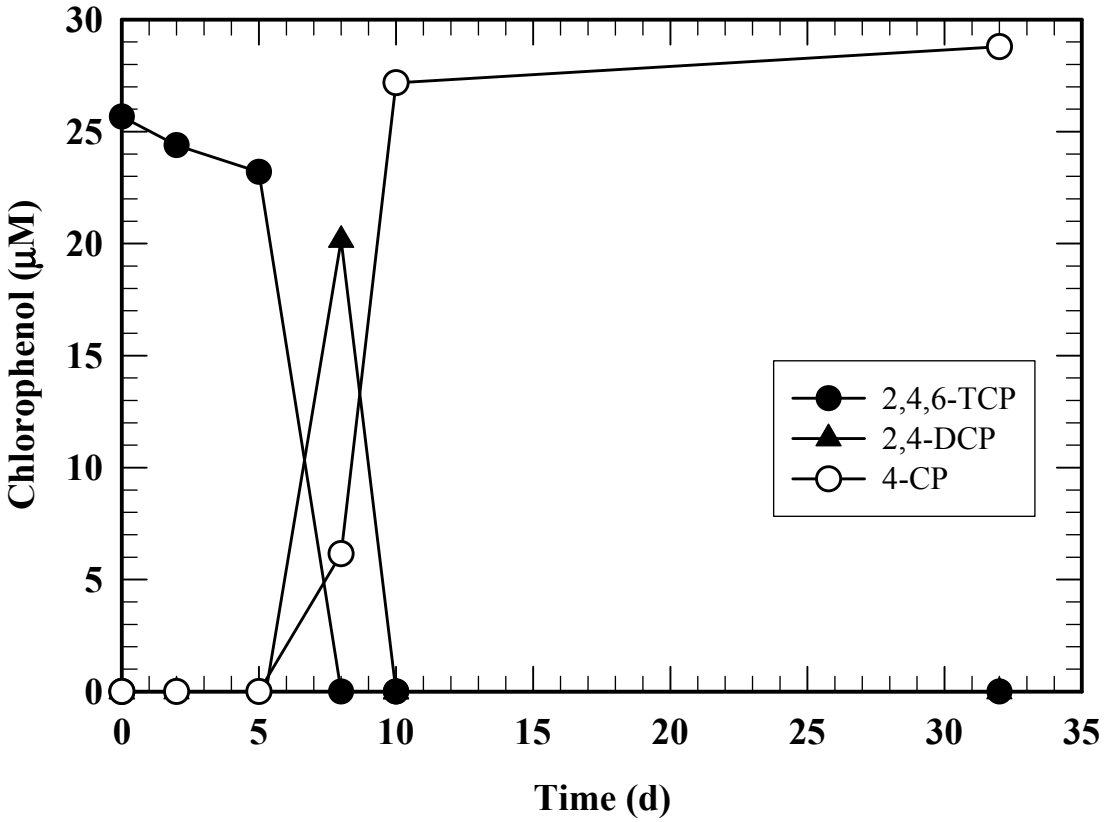


Figure 8.4. Reduction of 2,4,6-TCP to 4-CP with 2,4-DCP as an intermediate by strain Viet1 over a 32 d time period.

Reduction of Plant-Sequestered 2,4-DCP by strain Viet1

Isolate Viet1 was used to assay biologically available plant-sequestered 2,4-DCP by monitoring the stable end-product of metabolic dechlorination, 4-CP. *L. minor* (6.5 g) was exposed to 20 mg/L of 2,4-DCP in 400 mL media for 67 hr to achieve high a concentration of 2,4-DCP in plants. Plants were exposed to 2,4-DCP at pH 6 in order to achieve accelerated mass loading and exposure concentration was not considered to cause toxic effects in plants based on results previously outlined for 4-Cl-2-FP. The 2,4-DCP concentration profile for plant exposure is shown in Figure 8.5 where plants rapidly accumulated 2,4-DCP. All 2,4-DCP removed from aqueous phase was considered to be internal to plants based on ^{14}C-data presented in Figures 5.6 and 5.7. A concentration of 3.8 μmol 2,4-DCP / g plant was reached after 67 hr. Plants were removed from the reactor, rinsed, blotted dry, and frozen at -80ºC for 4 hr. Plants were then ground to a coarse powder and measured into aliquots (0.50 ± 0.01 g). Plants were placed in 90 mL serum bottles containing 30 mL anaerobic mineral salts media in an anaerobic glove box. Reactors were amended with excess lactate (5 mM) and 2 % v/v inoculum of an actively dechlorinating culture of strain Viet1. Six reactors received inoculum, three were active-Viet1 reactors and three reactors were autoclaved to serve as abiotic controls (autoclaved reactors). One reactor was not amended with strain Viet1 (no-Viet1 reactor) to serve as a control to determine if microorganisms naturally associated with *L. minor* play a role in chlorophenol degradation in anaerobic systems. Therefore, reactors contained ~ 1.9 μmol of plant-sequestered chlorophenol and total molar concentration was ~ 63 μM (values are presented in subsequently in Table 8.1).

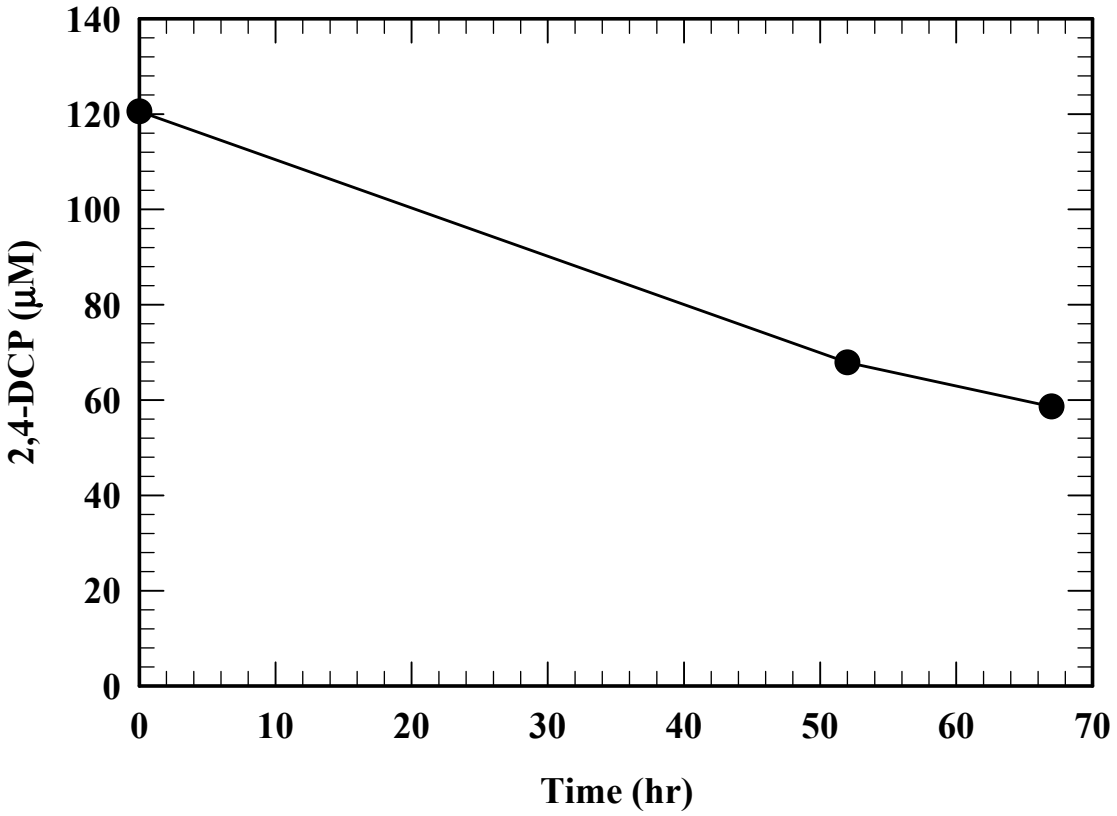


Figure 8.5. Uptake of 2,4-DCP by 6.5 g *L. minor* over 67 hr. A concentration of 3.8 μmol 2,4-DCP / g plant was achieved.

Reactors were sampled at 0.02, 6, 36, and 118 d and concentrations of 2,4-DCP and 4-CP were assessed. Initial samples were taken approximately ten minutes after addition of strain Viet1 and 30 minutes (0.02 d) after addition of plants to reactors to assess initial contaminant concentrations. Initial contaminant concentrations ranged from 4.8 – 5.7 μM 2,4-DCP in active-Viet1 reactors and 3.8 – 6.0 μM 2,4-DCP in autoclaved reactors, while 4-CP was not initially detected in any reactor. Contaminant recovered as aqueous 2,4-DCP during the initial sample was 5.9 – 9.3 % of total material in reactors. These data indicated that 2,4-DCP accumulated internal to plants was released upon addition of ground plants to media. The interval between addition of plants to reactors and removal of the initial sample was considered to be too small for significant microbial activity to occur to cleave sugar moieties conjugated to chlorinated phenols.

Samples taken after 6 d of exposure showed a significant increase in 2,4-DCP concentration in active-Viet1 and autoclaved reactors. Contaminant profiles for representative active-Viet1 and autoclaved reactors are presented in Figure 8.6. Data for the active-Viet1 system show that 2,4-DCP concentration increased from 5.3 μM at 0.02 d to 23.6 μM at 6 d. In the autoclaved system, 2,4-DCP concentration only increased from 5.9 μM at 0.02 d to 9.2 μM at 6 d. Significantly more 2,4-DCP accumulated in active-Viet1 reactors than autoclaved reactors by 6 d. Parallel increases in 2,4-DCP

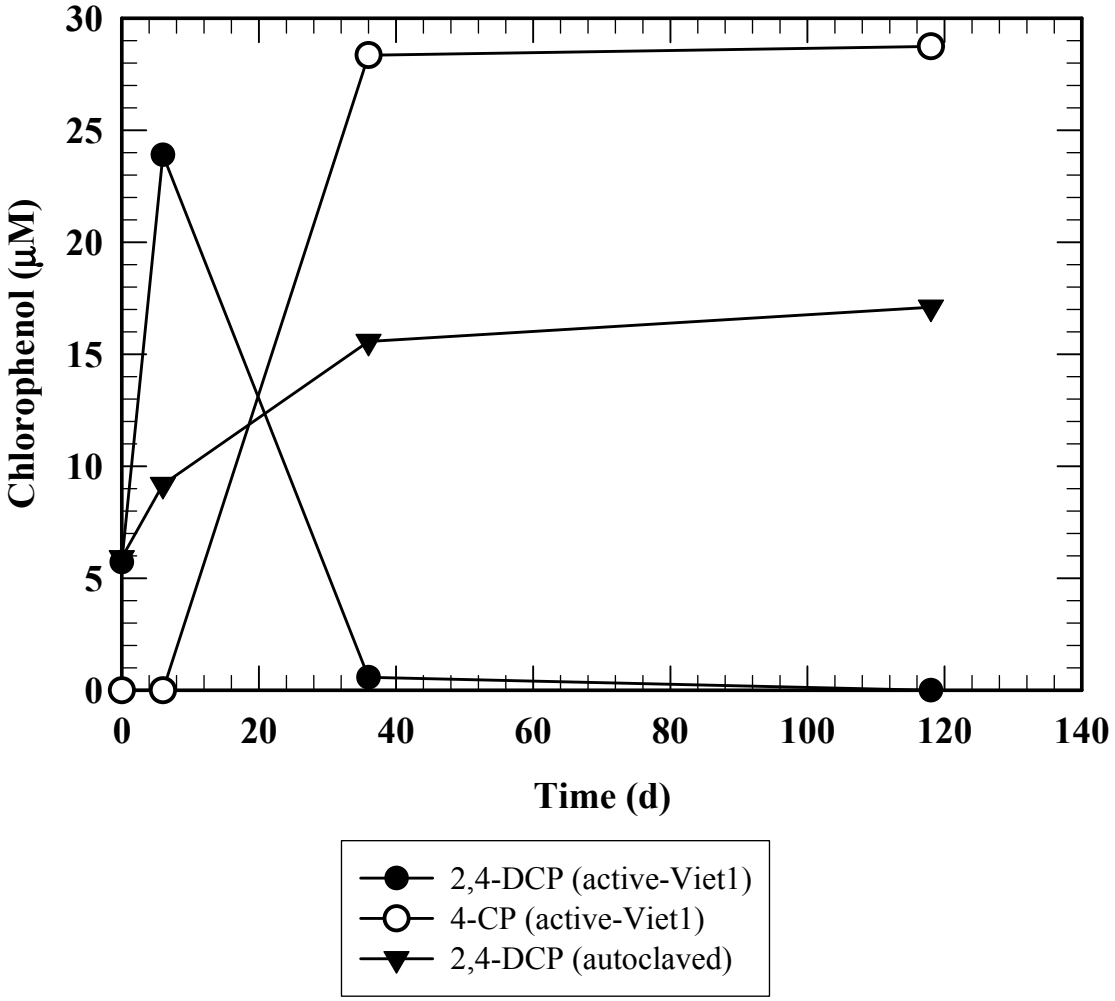


Figure 8.6. Representative data are provided for degradation of plant sequestered 2,4-DCP by active-Viet1 and autoclaved reactors. 2,4-DCP was degraded in active-Viet1 reactors and no degradation was observed in autoclaved reactors.

concentrations to those presented in Figure 8.6 were observed with triplicate reactors for active-Viet1 and autoclaved systems and data for all reactors are presented in Table 8.1.

After 36 d of exposure, 2,4-DCP concentrations in active-Viet1 reactors were reduced to very low levels (0.0 – 1.1 μM 2,4-DCP) and a corresponding production of a significant mass of 4-CP (22.1 – 29.3 μM 4-CP) was observed. Concentrations of 0.6 μM 2,4-DCP and 28.4 μM 4-CP were observed after 36 d in the active-Viet1 example data provided in Figure 8.6. The average 4-CP concentration increased minimally between 36 d and 118 d in active-Viet1 reactors, where data for the example system in Figure 8.6 did not show a significant increase. Reactors for active-Viet1 reactors showed parallel reduction of 2,4-DCP and production of 4-CP within the triplicate and data for all reactors are presented in Table 8.1. Depletion of 2,4-DCP and accumulation of 4-CP in active-Viet1 reactors was attributed to chlororespiring processes of strain Viet1. No 4-CP production was observed in autoclaved reactors. 2,4-DCP concentrations increased to concentrations of 16.1 – 17.2 μM by 118 d (Figure 8.6) in autoclaved reactors and parallel processes were observed within the triplicate (Table 8.1).

A comparison of total material recovered in active-Viet1 and autoclaved reactor systems for 0.02, 6, 36, and 118 d is presented in Figure 8.7. Total material recoveries increased with time in both active-Viet1 and autoclaved systems, and significantly more contaminant was recovered in active-Viet1 systems than autoclaved systems. After 118d, 43.5 (±1.4)% of contaminant in active-Viet1 reactors was recovered as 4-CP or 2,4-DCP. In autoclaved systems, 26.1 (±1.0)% of contaminant was recovered as 2,4-DCP by 118 d.

Table 8.1. Data provided for triplicate reactors in active-Viet1 reactors, autoclaved reactors and one active, no-Viet1 control

		Active Viet1 Reactors			Autoclaved Reactors			No Viet1 Reactor
		1	2	3	1	2	3	1
2,4-DCP (μM)	0.02 d	4.8	5.3	5.7	6.0	3.8	5.9	3.1
	6 d	15.8	20.6	23.9	6.6	9.7	9.2	13.7
	36 d	0.0	1.1	0.6	11.3	15.5	15.6	15.5
	118 d	0.0	0.6	0	16.1	17.2	17.1	3.3
4-CP (μM)	0.02 d	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	6 d	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	36 d	29.3	22.1	28.4	0.0	0.0	0.0	0.0
	118 d	25.5	27.4	28.7	0.0	0.0	0.0	17.2
Total μM in Reactor		60.9	62.8	65.1	64.4	64.0	64.8	62.6
Percent Recovered at 118 d		41.9	44.5	44.2	25.1	26.9	26.4	32.8

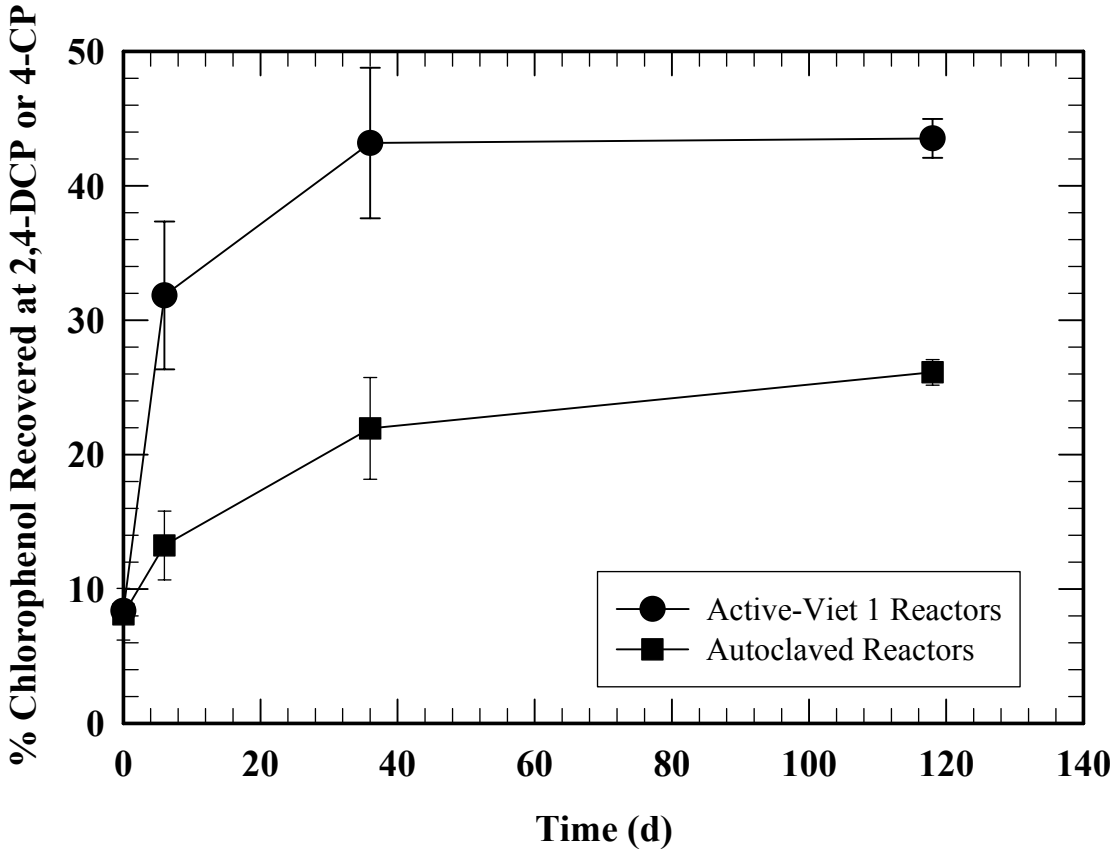


Figure 8.7. Percent of chlorophenol in plants recovered as 2,4-DCP or 4-CP in autoclaved and active-Viet1 reactors.

Therefore, a 17.4% difference between recoveries in active-Viet1 and autoclaved systems was observed.

One active reactor (no-Viet1 reactor) was not amended with isolate Viet1 and served as a control to determine if microorganisms naturally associated with *L. minor* play a role in chlorophenol degradation. Significant levels of 2,4-DCP accumulated in the media (Figure 8.8) and accumulation of 2,4-DCP was similar to that of active-Viet1 systems at 6 d and paralleled that of autoclaved-reactors in samples at 36 d (Table 8.1). No 4-CP was produced in the no-Viet1 reactor by 36 d. After 118 d, significant production of 4-CP was observed in the no-Viet1 control and concentration of 2,4-DCP had decreased correspondingly as presented in Figure 8.8. Material recovery for the no-Viet1 control reactor was 32.8%, a value which was significantly greater than autoclaved reactors and significantly less than active-Viet1 reactors.

Discussion

Experimental results have established that active plants take up and sequester halogenated phenols. This study addressed the fate of contaminants sequestered in plants after plant death. A significant portion of plant-sequestered 2,4-DCP was released into media after plants were frozen and ground to a powder. Only 3.1 – 6.0% of sequestered 2,4-DCP was released 30 min after ground-plants were placed in the media. Plants continued to release 2,4-DCP with time in autoclaved, active-Viet1 and no-Viet1 systems. Aqueous concentrations of 2,4-DCP gradually accumulated in autoclaved

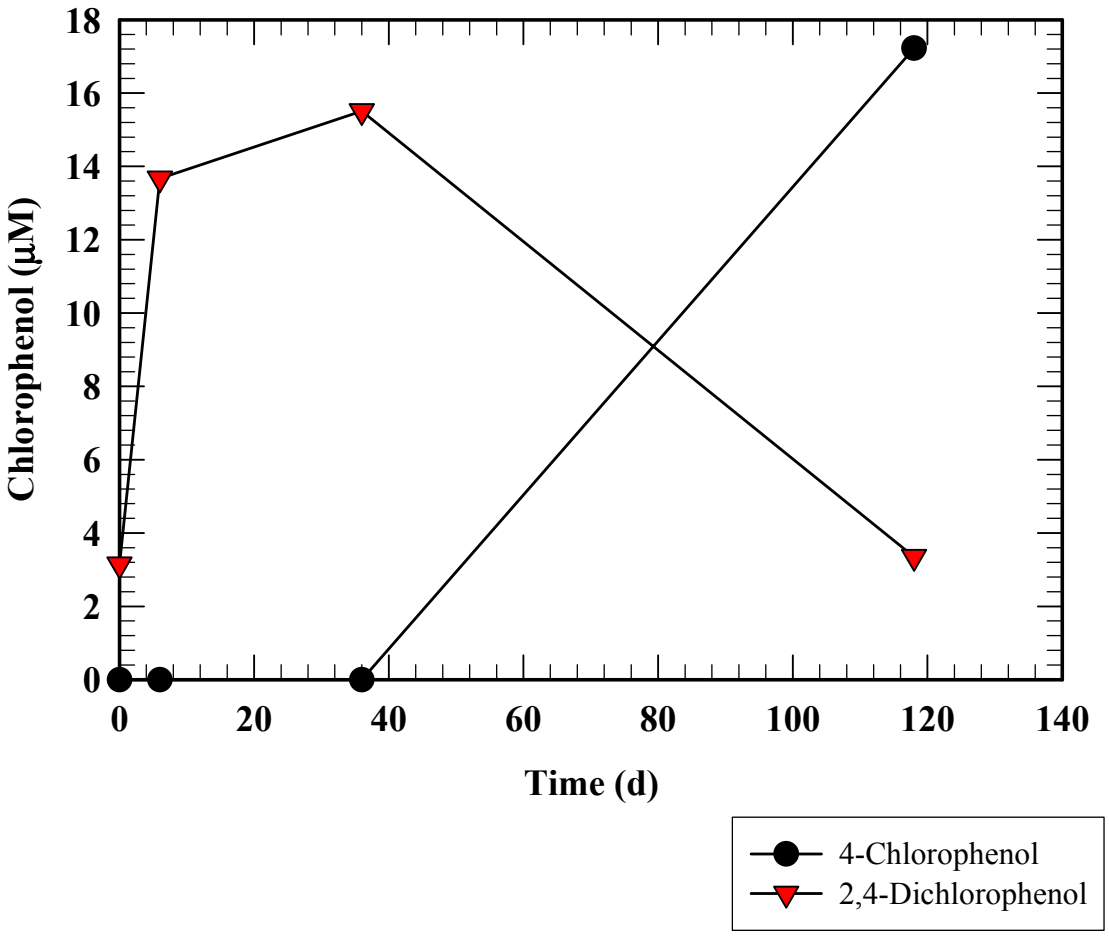


Figure 8.8. Production of 4-CP was observed in no-Viet1 control reactor after 118 d.

reactors, with more rapid accumulation occurring over earlier time points and no significant increase after 26 d (Figure 8.7). After 118 d, 26.1 (±1.0)% of plant-sequestered 2,4-DCP was released into the media in the autoclaved reactors. The increase in 2,4-DCP in autoclaved systems was attributed to a slow release of contaminant which had partitioned into plants but had not been enzymatically processed by plants.

Release of 2,4-DCP from plants in autoclaved systems corresponded to data presented by Day (2002) which quantified mass of 2,4-DCP internal to *L. minor*. Data presented by Day (2002) used four aqueous phase concentrations (21, 48.8, 50.5 and 98 μM) of 2,4-DCP for plant exposure and quantified 2,4-DCP and metabolites internal to plants. 2,4-DCP internal to plants which had not been enzymatically processed by plants (i.e., the conceptual equivalent of C_i as outlined in the conceptual model in Chapter 7) was represented as the fraction of contaminant internal to plants which had not been processed by plants or $F_{unprocessed}$. Data were presented by Day (2002) in terms of percent conversion (i.e., percent of total contaminant in the reactor that had been accumulated by plants). Data provided by Day (2002) are shown in Figure 8.10 where $F_{unprocessed}$ is plotted versus % conversion. A first-order relationship existed between $F_{unprocessed.}$ High values of $F_{unprocessed}$ (~100%) were observed at low values of % conversion. Low values of $F_{unprocessed}$ (<20%) were observed for high levels of conversion and $F_{unprocessed}$ dropped near zero as systems approached 100% conversion. Concentration of unprocessed contaminant in plants was assessed for 12 – 100% conversion and $F_{unprocessed}$ ranged from 3 – 107%.

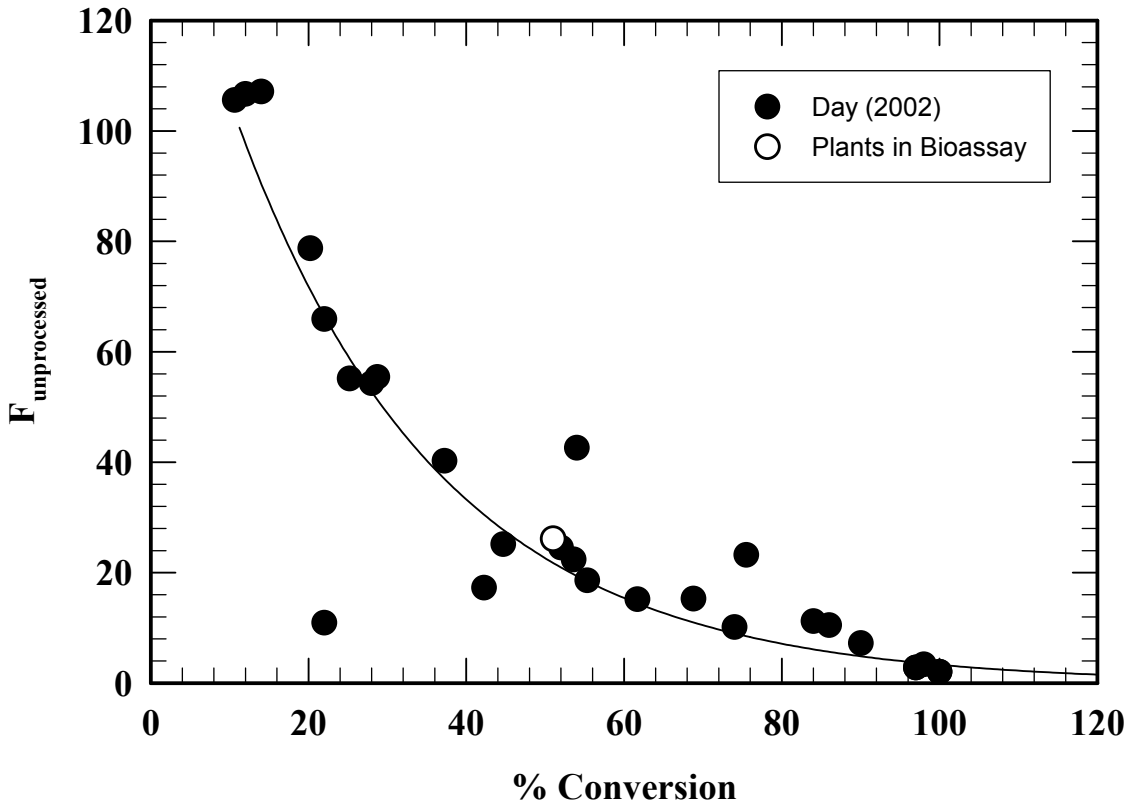


Figure 8.10. Data presented by Day (2002) which quantified 2,4-DCP internal to plants exposed to 21, 48.8, 50.5 and 98 μM 2,4-DCP are presented in terms of % conversion.

Plants used in this study were at 51% conversion and 26.1 (±1.0)% of contaminant was released into media after 118 d. The data point for plants used in this study and % contaminant recovered in autoclaved reactors is plotted with the data from Day (2002) in Figure 8.10. In studies reported by Day (2002) values for $F_{unprocessed}$ for the three concentrations tested after ~52 - 54% conversion were 23.1 (±1.9), 25.5 (±2.9), and 44.2(±4.8)% with error reported for triplicate samples. The value for fraction of contaminant released into media by plants after 118 d falls within the range of $F_{unprocessed}$ measured by Day (2002), indicating that 2,4-DCP released by plants in autoclaved systems was unprocessed contaminant sequestered internal to plants.

It is important to note that differences in plant density and contaminant exposure concentration exist between results provided by Day (2002) and those discussed herein. Plant density used by Day (2002) was 35 - 38 g plant/L and plant density used herein was 16.3 g/L. In addition, plants used herein were exposed to a higher concentration of 2,4-DCP (120 μM) than those used by Day (2002). As a result, plants used in this work contained significantly more contaminant than those examined by Day (2002) where 3.8 μmol/g was the concentration examined herein and the maximum concentration examined by Day (2002) was 1.3 μmol/g. These differences between plant density, exposure concentration and total contaminant accumulated could play a role in distribution and concentration of products and parent contaminant internal to plants.

Fraction of contaminant released into media in autoclaved systems were compared with $F_{unprocessed}$ measured for uptake of 4-Cl-2-FP using ^{19}F NMR presented in Chapter 5. ^{19}F

NMR measurements of plants exposed to 6.8 μM 4-Cl-2-FP (Figure 7.15) provided $F_{unprocessed}$ values of 6.5% at 30% conversion and 8.1% at 53% conversion. In the 13.1 μM 4-Cl-2-FP system (Figure 7.14) $F_{unprocessed}$ values were 10.4% at 18% conversion, 5.1% at 31.3% conversion and 7.9% at 70.9% conversion. Assessment of 4-Cl-2-FP internal to plants using ^{19}F NMR provided significantly lower quantities of unprocessed materials than results provided for 2,4-DCP by Day (2002). In addition, $F_{unprocessed}$ values were significantly lower than fraction of contaminant released into media in autoclaved systems. The reason for differences between ^{19}F NMR results, results presented by Day (2002) and values for $F_{unprocessed}$ are attributable to variations in plant density, contaminant structure and possible inhibitory effects.

Significantly more 2,4-DCP sequestered in plant material was released in microbially active-Viet1 systems than in autoclaved systems. After 6 d, 15.8 – 23.9% of plant sequestered 2,4-DCP was released in active-Viet1 systems, whereas 6.6 – 9.2% of plant sequestered 2,4-DCP was released in autoclaved systems. Therefore, significantly more 2,4-DCP was released in active-Viet1 systems than autoclaved systems after short-term exposure. After 118 d, 26.0 (±1.0)% of plant sequestered 2,4-DCP was recovered in autoclaved systems; however, in active-Viet1 systems 43.5 (±1.4)% sequestered 2,4-DCP was recovered in media as 2,4-DCP or 4-CP. Equilibrium-sorption to plants was considered as a possible explanation for release of additional contaminant in active-Viet1 systems where 2,4-DCP was converted to 4-CP than in autoclaved systems where 2,4-DCP was not converted to 4-CP. However, extrapolation from the isotherm for 2,4,5-TCP (Figure 4.4) indicated that sorption could only account for a trivial variation (~4%)

in 2,4-DCP released where a significant variation in 2,4-DCP was actually observed (17.1%).

The additional 2,4-DCP observed at early time points in active-Viet1 systems and the higher total chlorophenol concentration observed at the end of the study in active-Viet1 systems was considered a result of microbial cleavage of conjugated metabolic products produced by plants in addition to release of enzymatically unprocessed contaminant which had partitioned into plants. Cleavage of glycoside linkage by microorganisms was established in experiments conducted by Day (2002) which were designed to assess chlorophenol metabolite stability in reactors containing *L. minor*. Synthesized 2,4-dichlorophenol-glycoside and 2,4-dichlorophenol-malonyl-glycoside were added to triplicate reactors containing *L. minor*. For both compounds, the ester linkages were rapidly broken and 2,4-DCP accumulated in the media. In 2,4-dichlorophenol-malonyl-glycoside systems, 2,4-dichlorophenol-glycoside was released as an intermediate product. In both systems, degradation of 2,4-dichlrophenol-glycoside produced 2,4-DCP which was subsequently taken up by plants. In addition, cleavage of the glycoside linkage was observed in plant-free controls (Day, 2002). A lag-phase of ~30 hr was observed in plant-free systems indicating that cleavage of conjugated moieties before the initial sample taken after 30 min was unlikely. These results demonstrated the ability of ambient and plant-associated microorganisms to readily cleave malonyl and glycoside linkages, producing 2,4-DCP. Results provided by Day (2002) were produced in an oxic environment, however rapid cleavage of conjugated moieties in anaerobic environments

was expected in anaerobic environments since glycosyl hydrolase enzymes are present in aerobic and anaerobic environments (Himmel *et al.,* 2001; Ikan, 1999).

An active microbe control without isolate Viet1 amendment was used to determine if native microorganisms played a role in chlorophenol degradation. Because plants were frozen at -80°C and were not sterilized before being added to reactors, microorganisms associated with plants were present in reactors, although microbial activity was low at initial time points. After 6 d, more 2,4-DCP was released from plants in the no-Viet1 control than autoclaved reactors. However, 2,4-DCP concentration was less than that of active-Viet1 reactors possibly indicating some microbial cleavage of conjugated metabolites, without high levels of activity observed in strain Viet1 amended reactors. Degradation of 2,4-DCP in the no-Viet1 reactor did not parallel active-Viet1 reactors, where no production of 4-CP was observed after 36 d in no-Viet1 reactor and nearly all 2,4-DCP was degraded in strain Viet1 amended reactors. Partial degradation of 2,4-DCP occurred in the no-Viet1 control after 118 d, with significant production of 4-CP (17.2 μM) and low concentrations of 2,4-DCP remained (3.3 μM). These results indicated that degradation of plant-sequestered 2,4-DCP was possible in anaerobic systems without strain Viet1 amendment.

The microorganism strain Viet1 was used because of its ability to dechlorinate 2,4-DCP at the ortho position and lack of ability to access the chlorine in the 4-position, thus providing a stable endpoint for assessment of total 2,4-DCP released from plants. Using the assumption that 4-CP was a stable endpoint in the anaerobic-plant systems, results

indicated that 43.5 (±1.4)% of plant-sequestered 2,4-DCP was released from plants after 118 d. These results indicated that a significant portion of plant-sequestered 2,4-DCP was retained in the plant material for an extended time period (118 d) in anaerobic systems after plant death. However, results from no-Viet1 controls that showed dechlorination of 2,4-DCP after 118 d demonstrated that dechlorinating microorganisms other than strain Viet1 were present in plant material added to reactors. Because 2,4-DCP dechlorinating organisms were present in plant material, the possibility exists that 4-CP degrading microorganisms were also present in plant material. However, phenol, the most likely degradation product of 4-chlorophenol, was not observed in any sample. Therefore, an unlikely possibility existed that mass balance calculations based on the assumption that 4-CP was a stable end-product were not valid.

Results from autoclaved reactors showed that contaminant which has partitioned into plants but had not been enzymatically processed (C_i) was slowly released into surrounding media upon plant inactivation. In addition, results from active-Viet1 reactors showed that a significant fraction of enzymatically processed contaminant (C_p) is readily released into media upon plant inactivation. Following release into media C_p was broken down by microorganisms and parent contaminant was returned. However, only 43.5 (±1.4)% of plant-sequestered 2,4-DCP was released from plants over 118 d indicating that plants may provide a reservoir for long-term sequestration of contaminants. Finally, observation of degradation of 2,4-DCP in no-Viet1 reactor indicated that plant-associated microorganisms have the ability to reduce chlorinated organics.

CHAPTER 9

SUMMARY OF RESULTS

A summary of results describing factors which affect and control contaminant uptake by *L. minor* is presented. Factors which affect rate uptake of halogenated phenols by *L. minor* have been systematically examined in order to develop an understanding of processes controlling plant interactions with contaminants. Results describing dependence of partitioning into plants on protonated form were integrated with those which demonstrate the link between uptake by plants and plant activity and the dependence of uptake rate on speciation in the cytosol. Finally, the overall role of aquatic plants in the fate of contaminants in natural and engineered environments was explored by comparison of relative rates of uptake by aquatic plants with various degradation scenarios.

Uptake of Halogenated Phenols by Aquatic Plants

Uptake of 14 chlorinated, fluorinated, and brominated phenols was examined to determine effects of halogen positioning, type and number on rate of contaminant uptake. Results showed that substitution of fluorine for chlorine substituents did not systematically affect rate of uptake by *L. minor,* and in many cases, no change in uptake rate was observed between fluoro- and chloro- substituted phenols (Figures 4.17 and

4.21). The number of halogen substituents was not a controlling factor in halogenated phenol uptake. A wide range of uptake rates were observed for tri-substituted phenols (0 – 1.034 hr^{-1}). Di-halogenated phenols also provided a wide range of uptake rates (0.246 – 1.015 hr^{-1}) which overlapped with the range observed for tri-halogenated phenols. Rate of uptake of mono-halogenated phenols (0.797 – 1.106 hr^{-1}) was also within the range observed for di- and tri-halogenated phenols. Positioning of halogen substituents was observed to have a significant effect on uptake rate, where phenols substituted in the 2 and 6 position were observed to be taken up more slowly than phenols with other substitution patterns (Figure 4.21). Phenols with halogens in the 2,4,6 arrangement were not taken up by *L. minor* (Figure 4.20).

Uptake of a single halogenated phenol (2,4,5-TCP) was examined in further detail to gain an enhanced understand of processes that influence plant-contaminant interactions. Results from experimentation with 2,4,5-TCP demonstrated that uptake of contaminants was a passive process which was driven by abiotic partitioning of contaminants into plants. Rate of contaminant uptake was linearly dependant on fraction of contaminant which was protonated in aqueous phase, *f*, and independent of total-species concentration. Results showed that contaminant uptake rate increased linearly with increasing fraction TCP in protonated form (*f*). The relationship between *f* and contaminant uptake rate was quantified using the term κ as was presented in Figure 6.14. These results demonstrated that the protonated form of contaminant was the form available for partitioning into plants. Limited quantities of contaminant partitioned into plants when only a small fraction of the contaminant was in the available form (i.e., high aqueous pH values) and

rapid partitioning into plants occurred when the majority of the contaminant was in the available form (i.e., low aqueous pH values). The concept of protonated form partitioning into organic material represented by plants is paralleled by protonated fraction dependant partitioning into organic matter which has been detailed for soil and sediment systems.

Contaminant uptake rate has been positively linked to plant activity (Figures 6.9 and 6.10). These results demonstrate the importance of incorporating plant activity in studies of environmental fate of contaminants and phytoremediation design. In addition, the positive link between plant activity and contaminant uptake rate indicated that contaminant uptake is a process actively influenced by plant activity. Additional data compared rate of contaminant uptake by multiple halogenated phenols in a system where fraction protonated in aqueous phase was greater than 0.9, thus eliminating availability in aqueous phase availability as a variable. No correlation was observed between contaminant uptake rate, k, and parameters typically used to describe abiotic partitioning into organic material such as K_{ow} or molecular connectivity indices. Results indicated that rate of abiotic partitioning into plants did not control rate of contaminant uptake when phenols were fully available for partitioning into plants (i.e., protonated in aqueous phase). These results were in contrast with previous research where Briggs and co-workers (1987) and Burken and Schnoor (1998) provided evidence for hydrophobicity-driven abiotic partitioning of contaminants into terrestrial plants with a Gaussian relationship between log K_{ow} and TSCF.

Upon further examination of factors that affect contaminant uptake rate among various halogenated phenols, a comparison of contaminant uptake rate with respective pK_a values demonstrated a shifting order relationship, where k increased linearly between pK_a values of 6.04 and ~8 (Figure 4.28). A plateau value for k was observed for contaminants examined with pK_a values greater than ~8.0. This analysis indicated a relationship between contaminant uptake rate and contaminant speciation, where variations in k ceased at pK_a values greater than 8.0. The range of pK_a values over which k was a variable (6.0 – 8.0) bracketed typical cytosolic pH values of 7.0 – 7.5 reported for plant systems (Gout *et al.,* 1992). Cytosolic pH depends on growth conditions (*e.g.*, a change of 0.4 pH units was observed in *Arabidopsis* sp. under various growth conditions (Scott and Allen, 1999)) and cytosolic pH was assumed to be influenced by disassociation of halogenated phenols internal to plants.

A representative pH of 7.0 was selected to examine processes that influence uptake of contaminant by aquatic plants. Values for pK_a ranged from 6.04 to 10 for halogenated phenols examined. Therefore, contaminant speciation internal to plants at the cytosolic pH value of 7.0 was variable among halogenated phenols tested. In particular, contaminant speciation internal to plants varied widely for halogenated phenols with pK_a values which ranged from 6 to 8. The pK_a range over which contaminant speciation at pH 7.0 varied most widely (6.0 – 8.0) was the pK_a range over which k values also showed variation as was shown in Figure 4.28. For pK_a values where contaminant speciation at pH 7.0 varied little, no variation in k values were observed (Figure 4.28). Observations of variations in pK_a with contaminant uptake rate were considered in the

same manner as results which demonstrated that fraction protonated in aqueous phase, f, controlled partitioning into plants. Speciation internal to plants was considered to be a critical variable, and was quantified using fraction in protonated form in the cytosol or $f_{cytosol} = (K_a/10^{-7} + 1)^{-1}$).

A correlation between uptake rate coefficient, k (hr^{-1}) and fraction in the protonated form at pH 7.0 ($f_{cytosol}$) was observed where k-values increased linearly as $f_{cytosol}$ increased (Figure 9.1). These results demonstrated that contaminant uptake rate increased as the fraction in the protonated form internal to plants increased. In further examination of this linear relationship, a linear regression of data in Figure 9.1 provided an R^2 value of 0.66, as shown with 95% confidence intervals. The t-statistic for the slope parameter generated a p-value < 0.0001, indicating that the slope parameter was known to be non-zero and demonstrated that the linear relationship between $f_{cytosol}$ and k values was valid. Therefore, a relationship existed where uptake rate coefficient, k (hr^{-1}) increased linearly with increasing fraction in the protonated form at pH 7.0 ($f_{cytosol}$). When the protonated form of the contaminant was predominant in cytosol, contaminant uptake was rapid. Alternatively, when the deprotonated form predominated in the cytosol, contaminant uptake was suppressed. The linear relationship between k and $f_{cytosol}$ implied that metabolic processing internal to plants depended on the abundance of the protonated form of the contaminant existing in the cytosol.

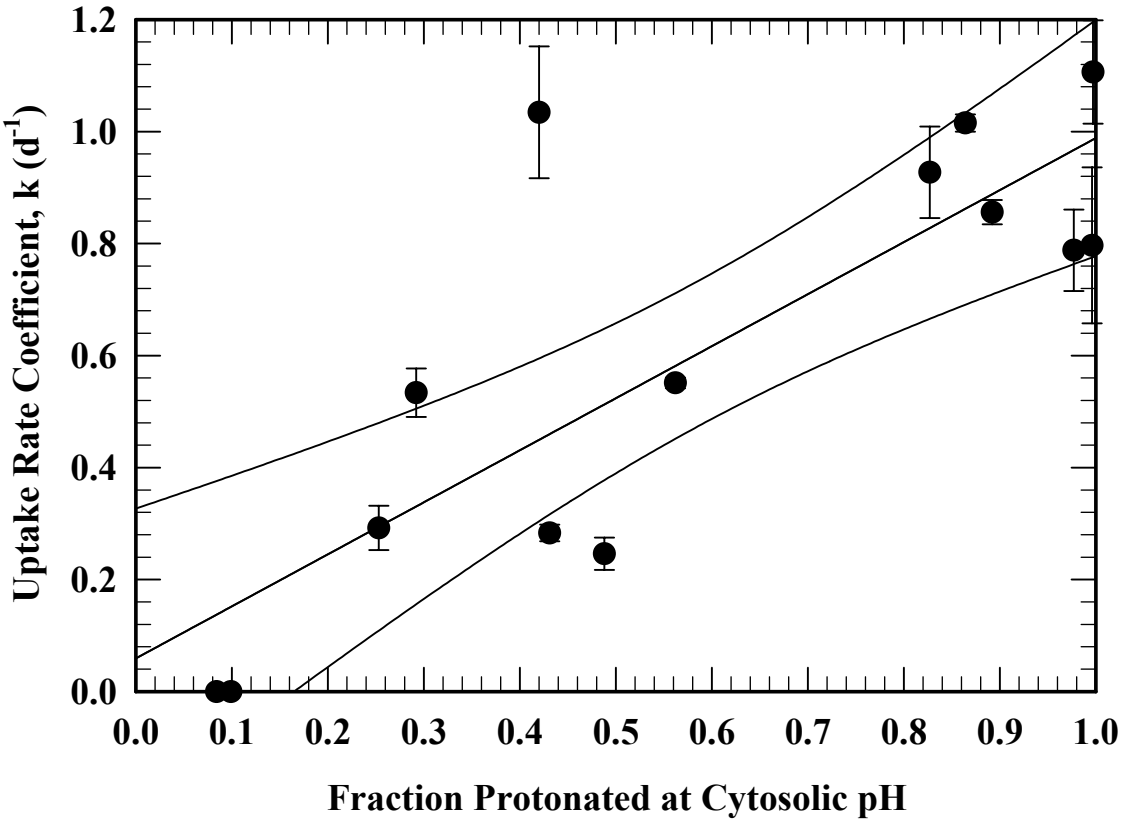


Figure 9.1. Uptake rate coefficient, k, for various halogenated phenols was linearly correlated with fraction protonated at cytosolic pH of 7.0. A linear regression of the data provided an R^2 value of 0.66 and is shown with 95% confidence intervals. The equation provided by the linear regression was $k = -0.929(\pm 0.191) * f_{cytosol} + 0.059(\pm 0.128)$.

The initial step in phenolic-contaminant processing by *L. minor* is conjugation of the hydroxyl moiety with a glucose molecule, a reaction mediated by a glucosyltransferase enzyme (Day, 2002; Leah et al., 1992). In plant systems, glycosyltransferase enzymes have been described as both soluble or membrane-bound enzymes (e.g., golgi bodies and endoplasmic reticulum) (e.g., Keegstra and Raikhel, 2001; Couthinho et al., 2003; Kreuz et al., 1996). In animal systems, it has been established that glycosyltransferase enzymes contain a substrate binding site internal to the endoplasmic reticulum (Meech and Mackenzie, 1997). It can be projected that a situation where glucosyltransferase enzymes are membrane bound and contain a substrate binding site internal to an internal membrane supports a scenario which dictates a dependence of k values on $f_{cytosol}$. Cytosol-speciation driven partitioning into an internal membrane such as the endoplasmic reticulum or golgi bodies is consistent with aqueous-phase-speciation driven partitioning of contaminants into plants as established previously (Tront and Saunders, 2004) and provides a possible explanation for shifting-order dependence of halogenated phenol uptake rate on contaminant pK_a values. In this scenario, contaminants that were ionized in the cytosol would exhibit limited partitioning into internal membranes and therefore would experience suppressed enzymatic processing due to lack of substrate availability to enzymes. When the contaminant was not enzymatically processed by plants, equilibrium was reached between cytosol contaminant and aqueous phase contaminant and contaminant was not continually removed from the external aqueous phase by plants. Conversely, contaminants that existed in the protonated form in the cytosol readily partitioned into internal membranes and were rapidly processed enzymatically by glucosyltransferase. When the contaminant was processed by the plant, the contaminant

was continually removed from aqueous phase. Membrane-bound glucosyltransferase enzymes provide a possible explanation for a shifting-order dependence of contaminant uptake rate on pK_a values and the linear relationship observed between k and $f_{cytosol}$.

Results showed that contaminant uptake was driven by the protonated form of the contaminant, indicating that contaminant uptake be an abiotic, passive process. These conclusions can be reconciled with results that showed a dependence of uptake rate on plant activity and results which outlined the dependence of contaminant uptake rate on rate of enzymatic processing by considering contaminant uptake rate and enzymatic processing in series. In the conceptual model outlined in Figure 7.7., abiotic partitioning of contaminant into plants was defined by the term κ (Figure 6.12) and equilibrium sorption to surfaces of plants was defined by a Freundlich isotherm (Figure 4.7). After the protonated form of the contaminant partitioned into the plant, the contaminant was enzymatically processed and packaged to reduce toxicity. Contaminants which existed in the protonated form in the cytosol readily partitioned into the endoplasmic reticulum and therefore were rapidly processed enzymatically by the gluconyltransferase enzyme. When the contaminant was processed by the plant, the contaminant was continually removed from aqueous phase. Contaminants which existed in the deprotonated form in the cytosol did not partition into the endoplasmic reticulum, and therefore were not processed by the gluconyltransferase enzyme. When the contaminant was not enzymatically processed by plants, equilibrium was reached between cytosol-contaminant and aqueous-phase contaminant and the contaminant was not continually removed from the aqueous phase by plants.

The relative rates of enzymatic processing of contaminants by plants and abiotic partitioning into plants played a crucial role in plant inhibition because the concentration of unprocessed contaminant internal to plants was the component toxic to plant systems. When the rate of enzymatic processing exceeded rate of abiotic partitioning into plants, contaminant did not accumulate internal to plants. This result was demonstrated with plant exposure to TCP in high pH systems where uptake occurred at rates slower than those in low pH systems. Slower uptake allowed systems to remain uninhibited because plants could enzymatically process the contaminant at a rate which did not allow for internal accumulation of the contaminant to toxic levels. Systems with decreased uptake rates often accumulated more mass than those with faster uptake rates because the contaminant did not get a chance to reach elevated concentrations internal to the plant and did not thereby cause toxic effects. When the rate of partitioning into plants exceeded the rate of enzymatic contaminant processing, a contaminant pool developed internal to plants. Increased uptake rates and the formation of a projected internal pool of contaminant were observed in low pH systems where rapid uptake caused toxic effects.

Inhibition in plant systems was quantified by incorporating the body burden concept typically used in aquatic toxicology into plant systems by assigning toxicity to contaminant internal to plants that had not been enzymatically processed by plants. Typical modes of quantifying inhibitory conditions are median effect concentration (EC_{50}) or median lethal dose (LD_{50}) which assess aqueous concentrations that cause an inhibitory effect or lethality in 50% of a population. Measurements of EC_{50} and LD_{50} do not account for parameters such as aqueous speciation, plant activity, rate of enzymatic

processing internal to plants or any other variations in rate of contaminant uptake by plants. This work provided an enhancement to typical modes of quantifying inhibition in aquatic systems by adding the critical plant residue term (CPR_{50}) which quantifies mass of parent contaminant internal to plants when 50% inhibition was observed. The CPR_{50} concept inherently accounts for rate of uptake because variations in rate of contaminant uptake and enzymatic processing are accounted for in calculation of CPR_{50}.

Inhibition of aquatic plants by halogenated phenols has been described as a primary function of proton-disassociation internal to plants and a resulting destruction of proton gradient. From data presented herein, it is clear that variations in pK_a of halogenated phenol play an important role in variations observed in toxic effects caused by halogenated phenols. If the pK_a is greater than cytosolic pH, rate of enzymatic processing will occur rapidly and little disassociation of the hydroxyl moiety will occur. Therefore, a limited number of protons will be released internal to plants and the proton gradient will not be disrupted. If the pK_a is less than cytosolic pH, rate of enzymatic processing will be slow and the halogenated phenol will accumulate internal to plants. Accumulation of halogenated phenol with a high pH will result in proton disassociation, the proton gradient will be disrupted and inhibitory effects will occur.

The strong dependence of toxicity on pK_a is consistent with previous research which reports an increase in chlorinated phenol toxicity with increasing chlorination up to three chlorines are present (Ensley *et al.,* 1997). Data showed toxicity increased in the following order: phenol < 4-CP < 2,4-DCP < 2,4,5-TCP = 2,4,5,6-TeCP = PCP for

Lemna gibba exposure in media with an undefined pH. The pK_a of chlorinated phenols studied by Ensley and co-workers (1997) decrease as toxicity increased where values for 2,4,5,6-TeCP and PCP are 5.2 and 4.3, respectively (SPARC) . Because rate of enzymatic processing depends on fraction protonated at cytosolic pH, it follows that 2,4,5,6-TeCP and PCP would not be rapidly processed internal to plants because only a small fraction of each contaminant internal to plants was protonated in the cytosol (pH ~ 7.0).

Sequestration and Metabolism of Halogenated Aromatics by Aquatic Plants

Two model plants, *L. minor* and *M. aquaticum*, were used to demonstrate that aquatic plants rapidly uptake and sequester halogenated aromatics. Example data presented in Figures 4.8 – 4.10 demonstrated the rapid nature of uptake of 2,4,5-TCP by *L. minor* and data presented herein detail the complex nature of factors which interact to control rate of contaminant uptake by plants. Fate of contaminants internal to plants and implications for long-term contaminant fate were explored using ^{14}C and fluorine-labeled contaminants. Use of ^{14}C labeled 2,4,5-TCP and 2,4-DCP demonstrated that contaminants were taken up and sequestered by plants. Results demonstrated that TCP depletion from aqueous phase was a result of net contaminant movement into plant tissue. TCP was rapidly sequestered internal to the plants and no net contaminant release into aqueous phase by plants over a 96 hr experimental time frame. Sequestration of contaminants and production of metabolites by aquatic plants was also established fluorinated analogs of chlorophenols and ^{19}F NMR. Analysis of liquid extracts of plants exposed to fluorinated-chlorinated phenols by ^{19}F NMR provided evidence for

sequestration of small quantities of parent material internal to plants. In addition, ^{19}F NMR analyses showed that several (3 – 6) metabolic products comprised the majority of chlorinated phenol sequestered internal to plants. Peaks representing metabolic products appeared down field of parent material demonstrating that substituents on the ring structure in the metabolic products contained less electron withdrawing abilities than those of the parent compound. In addition, peak splitting patterns in metabolic products and parent compounds were identical and therefore indicated that hydrogen bonding structure to the aromatic ring was not modified in the production of metabolic products.

Day (2002) demonstrated that *L. minor* conjugate 2,4-DCP and 2,4,5-TCP with glycoside, malonyl and apiose moieties by positively identifying three metabolic products. Quantification of conjugated products and parent material internal to plants allowed Day (2002) to achieve 50% (2,4,-DCP) and 70% (2,4,5-TCP) mass balance recoveries after all contaminant was removed from aqueous phase. Data presented herein enhance data presented by Day (2002) by demonstrating that 100% of material removed from aqueous phase was sequestered internal to plants. In addition, ^{19}F NMR evidence for six or more possible metabolites of 4-chloro-2-fluorophenol opens possibilities for additional metabolic products not identified by Day (2002) which may play an important role in plant processing of contaminants.

The integration of results outlined herein is presented in Figure 9.2 where movement of halogenated phenols in plant systems are represented with parameters which control processes illustrated. The dependence of abiotic partitioning into plants on protonated

form of 2,4,5-TCP is indicated by the fact that only the protonated form of TCP partitions into the plant where the parameter κ was used to define this interaction. The rate of enzymatic processing by plants was shown to proceed rapidly when contaminant was protonated internal to plants due to enzymatic processing occurring in the endoplasmic reticulum. Therefore, Figure 9.2 shows protonated halogenated phenol moving across the endoplasmic reticulum membrane and the initial step of enzymatic processing by plants, glycosidation, occurs within the endoplasmic reticulum. Enzymatic processing of contaminant into malonlyl and apiose conjugate moieties and packaging into vacuoles and cell wall were presented based on work presented by Day (2002) and Day and Saunders (2004). Therefore, the overall processing of contaminants by plants relies both on abiotic processes, where factors such as protonation must be considered, and on metabolically-driven factors where plant activity and rate of enzymatic processing must be considered.

Role of Aquatic Plants in Natural and Engineered Systems

To gain a deeper understanding of implications this work has in understanding overall fate of contaminants in plant systems, data comparing half-lives of 2,4,5-TCP in natural systems is presented in Table 9.1. Contaminant uptake half-lives were calculated for 2,4,5-TCP uptake by uninhibited *L. minor* (15g/L) ranged from 4.6 to 138 hr depending on exposure conditions. *M. aquaticum* (75 g/L) displayed half-life of 28.8 hr for uptake of 2,4,5-TCP. In comparison, reported half-lives of 2,4,5-TCP in microbial systems range from 23 – 690 days under aerobic conditions and 126 – 1,820 days under anaerobic

Figure 9.2. Integration of work presented herein where speciation in aqueous phase and internal to plant dictate partitioning into plants and endoplasmic reticulum and therefore control rate of contaminant uptake rate and processing.

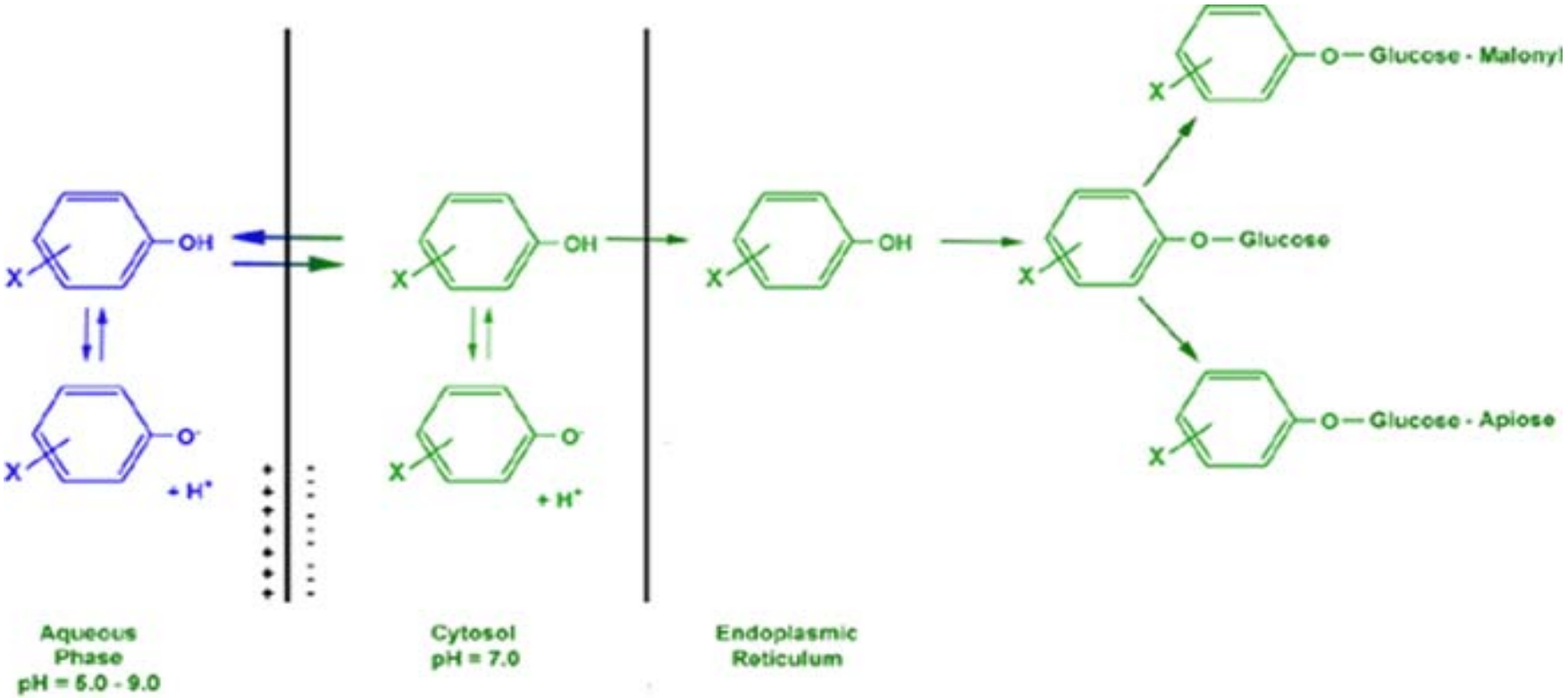


Table 9.1. Persistence of 2,4,5-TCP in environmental systems.

Process	$t_{1/2}$ (hr)	Conditions	Source
Aquatic Plant Uptake	4.6-138 28.8	*L. minor* (15 g/L) *M. aquaticum* (75 g/L)	Data Presented Herein
Abiotic Degradation	263	Experimental Conditions Used Herein	Data Presented Herein
Aquatic Photolysis[1]	0.5 336	Summer, 25ºC Winter, 18ºC	Howard *et al.,* 1991
Photooxidation[2]	66 – 3480 30.1 - 301	Water Air	Howard *et al.,* 1991
Anaerobic Microbial Degradation[3]	3028-43,690	-	Howard *et al.,* 1991
Aerobic Microbial Degradation[4]	552-16,560	-	Howard *et al.,* 1991
Hydrolysis	$>8x\ 10^6$	-	Howard *et al.,* 1991

[1]Based on photolysis rate constants for transformation and mineralization under summer and winter sunlight conditions at 25 and 18 degrees, respectively (Hwang *et al.,* 1986)

[2]Water: Scientific judgment based upon reported reaction rate constant for OH and RO2 with the phenol class (Mill and Mabey, 1985; Guesten *et al.,* 1981). Air: Scientific judgment based upon an estimated rate constant for the vapor reaction with hydroxyl radicals in air (Atkinson, 1987A)

[3]Scientific judgment based upon unacclimated anaerobic grab sample data for soil and groundwater (low $t_{1/2}$ from Gibson and Sulflita (1986); high $t_{1/2}$ from Baker and Mayfield (1980))

[4]Scientific judgment based upon unacclimated aerobic river die-away test data (Lee and Ryan, 1979)

conditions (Howard *et al.,* 1991; Mackay *et al.,* 1985). Reported half-lives for photooxidation of 2,4,5-TCP ranged from 0.5 – 336 hr (Howard *et al.,* 1991) and photoxidation measured in data presented in Figure 4.1 provided a half-life of 263 hr. Rate of uptake in plant systems is significantly faster than microbial degradation in aerobic or anaerobic systems. Plant uptake of contaminants was significantly faster than photooxidation in reactor systems used herein which were modeled after water-column structure light inputs. However, plants do not mineralize contaminants as was demonstrated by ^{14}C and ^{19}F NMR results and by results of others (Day, 2002). Plant interactions with contaminants remove contaminants from the water column or sediment system and sequester those contaminants in plant material. Mineralization of halogenated phenols did not occur after sequestration by plants, therefore photolytic degradation of plant-sequestered contaminant was assumed not to occur. Therefore, consideration of plant-contaminant interactions is critical to understanding the dynamics of water column and sediment systems. In addition, these results indicate that plants play a critical role in fate of contaminants in engineered and natural systems.

Future Research Needs

Fate of halogenated aromatics in plant systems has been examined using experimental and modeling efforts that have helped to achieve a better understanding of interactions between contaminants and aquatic plants. Results presented herein delineate the role of aquatic plants in sequestration of contaminants and indicate the capabilities and limitations of use of aquatic plants in natural and engineered treatment systems. Additional research is necessary to more fully understand the role of attenuation of contaminants by plants. Examination of additional classes of contaminants with variation in chemical and physical

properties will lead to an enhanced understanding of factors that influence uptake and sequestration of contaminants by plants. In addition, assessment of plant interactions with chemicals that contain functional groups and base-molecular structures of emerging micro-contaminants such as polyfluorinated compounds, heterocyclic moieties and hormonally active compounds will provide insight into the role of plants in attenuation of emerging contaminants. Additional efforts are necessary to extend the model presented to incorporate diffusive flux of contaminants into plants using measured values of internal mass of unprocessed contaminant. An important contribution to understanding interactions of contaminants with plants could be gained with additional modeling efforts. Utilization of results presented which describe factors such as effects of plant activity, inhibition, contaminant speciation in aqueous phase and internal to plants and rate of enzymatic processing shows promise for simplification of the system and enhancement of the mathematical description of uptake of contaminants by plants. An extension of results presented herein for quantifying parent contaminant and extractable metabolites using ^{19}F NMR shows promise for modeling uptake by plants as a diffusive process and would provide insight into factors that control contaminant uptake by plants. Preliminary results showed an increase in non-extractable form of contaminant with moderate toxicity. Therefore, examination of plant sequestered contaminants using solid-phase NMR in whole plant systems and high resolution NMR (HR-NMR) in cell extract systems would provide insight into formation of non-extractable metabolites by plants. Coupling production of bound components to toxicity and inhibition would provide an important contribution to enhancement of engineered systems. Finally, extending examination of availability and degradability of plant sequestered contaminant into environments that

more closely simulate natural and engineered systems holds promise for an enhance understanding of the role aquatic plants in those systems.

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VITA

Jacqueline Marie Tront was born in Buffalo, New York to Joseph and Elizabeth Tront in September, 1976. Shortly thereafter, Jackie moved with her family to Blacksburg, Virginia where she grew up with her sister Katie. After graduation from Blacksburg High School, Jackie enrolled as a student in the Civil Engineering Department at Georgia Institute of Technology. Jackie was very involved in campus life as an undergraduate through participation in social and professional activities. Internships that Jackie held while she was an undergraduate at Georgia Tech with Draper Aden Associates, St. John's River Water Management District and Browning Ferris Industries helped to shape her interests and professional goals. Jackie earned a Bachelor of Science degree in Civil Engineering with honor in Spring 1998 after which she enrolled as a graduate student in the Environmental Engineering program at Georgia Tech to pursue her doctoral degree. As a graduate student, Jackie was very involved in environmental engineering program activities and the environmental engineering student association, Association of Environmental Engineers and Scientists (AEES). She held a number of leadership positions in AEES and served as the association president in 2001-2002. The author also received a number of awards during her tenure at Georgia Tech in recognition of academic, leadership and service activities. Among the awards she received were the American Association of University Women Dissertation Fellowship, the Presidential Fellowship from Georgia Institute of Technology and was named the AEES Outstanding

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